LVII ANNUAL MEETING
of the ITALIAN SOCIETY
for VETERINARY SCIENCES (SISVET)

Ischia 2003

CONTENTS

Round Table: **Development of European educational strategies and definition of the design of veterinarian profiles identified by market needs for the year 2020: the VET 2020 Project**

Chairman: S. Cinotti (Bologna)

Main speakers

The role of Vet2020 Project on Quality of European Veterinary Education
T Fernandes 9–11

Development of European Educational Strategies: Design of Veterinarian Profiles Identified by Market needs for the Year 2020
P De Castro, S Zucconi 13–28

Quality Assurance: The Key for Amendments of the EU-Directive/s Regulating Veterinary Training in Europe
H Rodríguez-Martínez 29–44

Invited speakers

An Analysis of the Veterinary Profession in Italy – Characteristics and Prospects
P Bossi 45–46

Italian University System has to Match Educational Needs in Adherence to European Standards and Professional Scenario
G Penocchio 47–49

Workshops:

N.1 **Shellfish Farming: Technologies and Production**
G Prioli 51–56

Defense Mechanisms in Farmed Marine Molluscs
PG Tiscar, F Mosca 57–62

National and EU Pathologies and Norms in Mollusc Culture
G Ceschia 63–67

Bivalve Molluscs: Productivity in the Campania Region and Related Sanitary Aspects
A Anastasio, E Fariello 69–74

N.2 **Veterinary Toxicovigilance: Objectives, Means and Organisation in France**
G Keck, P Berny, F Buronfosse, X Pineau, E Vermorel, B Rebelle, T Buronfosse 75–82

Incidence of Poisonings in Domestic Carnivores in Italy
A Giuliano Albo, C Nebbia 83–88

Epidemiology of Intoxications in Italy
M Amorena, F Caloni, G Mengozzi 89–95

Regional Regulations Prohibiting the Use and Possession of Poisoned Lures
G Brajon 97–102

Laboratory Diagnostic Examinations in Veterinary Toxicology
M Giorgi, B Naso 103–106

N.3 **Follicular Waves in Cattle**
JF Roche 107–110

Manipulation of Ovarian Function for the Reproductive Management of Dairy Cows
WW Thatcher, JA Bartolome, A Sozzi, F Silvestre, JEP Santos 111–119

Production and Quality of Bovine Oocytes and Embryos
C Galli, R Duchi, G Crotti, P Turini, N Ponderato, S Colleoni, I Lagutina, G Lazzari 121–126

N.4 **Buffalo Milk: Its Properties, Dairy Yield and Mozzarella Production**
L Zicarelli 127–135

Economic Problems in the Buffalo Milk *filière*
F de Stefano 137–142

Buffalo Meat Production: Performance *infra vitam* and Quality of Meat
F Infascelli, S Gigli, G Campanile 143–148

Selected Short Communications and Sections

BIOLOGY & REPRODUCTION

Factors Influencing the Quality of Life of the Cat in its Relationship with Owners
S Adamelli, L Marinelli, S Normando, A Camperio Ciani, G Bono 149–151

Urinary Incontinence After Spaying in the Bitch: Incidence and Oestrogen-therapy
A Angioletti, I De Francesco, M Vergottini, ML Battocchio 153–155

Signalling Compartmentalization Involved in the Boar Sperm Acrosome Reaction
B Barboni, P Lucidi, N Bernabò, MG Pistilli 157–160

Ultrasonographic Study During Pregnancy of the Growth of an Encephalic Portion in the Canine Foetus
M Beccaglia, GC Luvoni 161–164

The Correlation Between Mast Cells and Some Inflammatory Mediators in the Bovine Endometrium
S Belluzzi, M Galeotti, CM Eutizi, C Castagnetti 165–168

Clinical Use of Twice Daily Injections of Buserelin Acetate to Induce Ovulation in the Mare
F Camillo, M Pacini, D Panzani, I Vannozzi, Al Rota, G Aria 169–172

Effect of Cryoprotectant Agents on the Potential Development of Sheep Preantral Follicles
G Capacchietti, S Cecconi, L Gioia, M Turriani 173–176

Quantitative Motor Unit Action Potential Analysis in Skeletal Muscles in Horses and Ponies
B Ciminaghi, M Albertini, M Costanzi, F Patrese, MG Clement 177–179

Pig Reactivity to Backtest and Growth During the First Three Months of Life
M Faustini, E Munari, V Russo, G Pastorelli, GL Restelli, S Scocca, D Vigo 181–183

DNA Fingerprinting Using Microsatellites to Solve a Parentage Testing in the Boxer Breed
F Gentilini, ME Turba, G Andreani 185–188

Neurotrophins and the Thymus: Morphological Analysis of Mice Carrying a Non-functional Mutation on the Trka and Trkb Genes
A Germanà, R Laurà, MB Levanti, G Montalbano, GP Germanà, F Abbate, E Ciriaco 189–192

Immunodiscrimination Between Native and Recombinant Somatotropin: a Possible Pathway?
A Guidi, G Iannone, L Castigliego, D Gianfaldoni 193–196

Occurrence of Two Different Mechanisms of Apoptosis in Cerebellar Granule Cells in Relation to the Specificity of Poly-ADP-ribose Polymerase-1 (PARP) Activation
L Lossi 197–200

2B Myosin Heavy Chain Isoform Expression in Bovine Skeletal Muscle
F Mascarello, L Maccatrozzo, M Patruno, L Toniolo, C Reggiani 201–204

Oocyte Maturation is Required for Correct Sperm Chromatin Rearrangement
M Mattioli, L Gioia, M Turriani, G Capacchietti, P Loi, G Ptak 205–208

Inhaled Carbon Monoxide (CO) Prevents Lung Oedema Induced by Endotoxic Shock
S Mazzola, M Forni, M Albertini, ML Bacci, B Ciminaghi, M Lavitrano, E Seren, MG Clement 209–212

Ghrelin in the Gastroenteric Tract of Birds: Immunoreactivity Expression
S Neglia, N Arcamone, V Esposito, G Gargiulo 213–215

ZOOTECHNICS & ANIMAL NUTRITION

Effects of Acetyl-salicylate Used in Post-calving of Dairy Cows
G Bertoni, E Trevisi, F Piccioli-Cappelli 217–219

Use of Two Different Energy-value Diets on Sarda Ewes During Late Pregnancy and Suckling
F Bovera, R Schettini, MI Cutrignelli, S Calabrò, F Infascelli, V Piccolo 221–224

Sensory Evaluation of Sea Bass (*Dicentrarchus labrax* L.) Fed Two Diets Differing in Fat Content
RM Lopparelli, S Segato, A Corato, L Fasolato and I Andrighetto 225–227

Survey of Ochratoxin A in Cereals from Puglia and Basilicata
M Muscarella, C Palermo, T Rotunno, V Quaranta, P D'Antini 229–232

First Pregnancies Carried to Term After Transfer of Vitrified Buffalo Embryos Entirely Produced *in vitro*
G Neglia, B Gasparrini, V Caracciolo di Brienza, R Di Palo, L Zicarelli 233–236

Effect of Total Replacement of Dietary Fish Meal by Plant Protein Sources on Early *post mortem* Changes in the Biochemical and Physical Parameters of Rainbow Trout
G Parisi, M de Francesco, F Médale, F Scappini, M Mecatti, SJ Kaushik, BM Poli 237–240

Effects of Dietary Conjugated Linoleic Acid (CLA) on Immunoglobulin Concentration in Sow Colostrum and Piglet Serum
R Rossi, G Pastorelli, V Bontempo, C Corino 241–244

Repeatability of Milk Clot Yield and Possibility of Reducing the Number of Controls Required to Identify the Individual Yield in the Buffalo
L Zicarelli, A Potena, L Amante, R Di Palo 245–247

FOOD INSPECTION

Detection of *Escherichia coli* Serotype 0157 in Beef Carcasses and Faecal Material
S Albonetti, M Trevisani, S Alonso Alvarez, R Rosmini 249–251

Setting up a PCR Based Method to Trace Animal Species in Processed Meat Products
MN Losio, ML Ferrando, P Daminelli, F Chegdani 253–255

Proposal of an Analytical Method for Determination of Residues of Organophosphorus Pesticides in Milk by GLC-NPD
G Pagliuca, T Gazzotti, E Zironi, N Pavoncelli, R Rosmini 257–259

Research on Bacteraemia in Cultured Rainbow Trout
A Panebianco, A Giuffrida, F Conte, G Ziino, A Minniti, L Iannuzzi 261–263

Efficacy of Certain Disinfectants Towards Enteroviruses: Kinetics of *in vitro* and *in vivo* Inactivation
E Pavoni, MN Losio, E Bonetti, E Cosciani Cunico, C Medici 265–267

Microbiological Characteristics of Hamburgers and Raw Pork Sausages, and Antibiotic-resistance of Isolated Bacteria
D Ranucci, D Miraglia, R Branciari, V D'Ovidio, M Severini 269–272

ANIMAL PATHOLOGY

Rare Tumours in Domestic Animals: A Lipid Cell Variant of Urothelial Carcinoma of the Urinary Bladder in a Cow and a Case of Vesical Carcinosarcoma in a Dog
G Borzacchiello, V Ambrosio, L Leonardi, R Fruci, P Galati, F Roperto 273–274

Aflatoxicosis and Vitamins A and E Supplementation in Sows: Immunological State of their Piglets
E Cabassi, R Di Lecce, E De Angelis, A Fusari, A Perillo, P Borghetti 275–277

Improved Detection of *Coxiella burnetii* in Cows Milk by Immunomagnetic Separation and PCR
F Capuano, AG Perugini, A Parisi, CO Montagna, M Nilvelli 279–282

Effect of Temperature on *Betanodavirus* Infection in SSN-1 Cell Line
S Ciulli, F Ostanello, M Battilani, S Prosperi, RP Hedrick 283–286

Evaluation of endogenous pig retrovirus expression and of tumorigenicity in nude mice
M Ferrari, A Corradi, PG Petronini, A Tosini, A Toniolo, C Robotti, R Moratti 287–290

Cloning and Expression of the Orf Virus F1L Gene: Possible Use as a Subunit Vaccine
L Gallina, A Scagliarini, S Ciulli, S Prosperi 291–293

Duplex Polymerase Chain Reaction Assay to Screen for Feline Herpesvirus-1 and *Chlamydophila spp.* in Mucosal Swabs from Cats
F Marsilio, B Di Martino, I Aguzzi, I Meridiani 295–298

Evaluation of Angiogenesis by Morphometric Analysis of Blood Vessels in Dysplastic and Neoplastic Lesions of Canine Gingiva
M Martano, P Maiolino, M Cataldi, B Restucci 299–301

Transmissible Spongiform Encephalopathy (TSE): Vaccinal Approach Using the Hamster Model
C Pollera, M Caramelli, ML Giannino, PA Martino, M Puricelli, C Casalone, P Gazzuola, G Poli 303–306

In vivo Model for the Evaluation of Molecules Active Towards Transmissible Spongiform Encephalopathies
W Ponti, M Sala, C Pollera, D Braida, G Poli, S Bareggi 307–310

Principal Endoparasitoses of Domestic Cats in Sardinia
M Porqueddu, A Scala, V Tilocca 311–313

Mycobacterioses: Emerging Pathologies in Aquarium Fish
M Prearo, RG Zanoni, B Campo Dall'Orto, E Pavoletti, D Florio, V Penati, C Ghittino 315–317

Antigenic Variability of Ovine Lentivirus Isolated in Italy
S Rosati, M Profiti, E Grego, ML Carrozza, M Mazzei, P Bandecchi 319–322

MEDICINE, SURGERY & PHARMACOLOGY

24-Hour Ambulatory Electrocardiography in the Dog
F Birettoni, F Porciello, F Rueca, G Fruganti 323–325

Plasma Thrombomodulin Levels in Dogs Naturally Infected with *Leishmania infantum*
P Ciaramella, L Cortese, M Corona, R Ambrosio, A Di Loria, A Persechino 327–330

Haemostatic Disorders in Dogs Naturally Infected by *Leishmania infantum*
M Corona, P Ciaramella, A Pelagalli, L Cortese, ME Pero, D Santoro, P Lombardi 331–334

MPA Inhibits Idarubicin Activity on Cu-Zn SOD and Catalase
L Crispino, R Ciarcia, S Montagnaro, R Pagnini, F Pacelli, S Florio 335–338

Breed Distribution of Canine Diabetes Mellitus in Italy
F Fracassi, M Pietra, A Boari, G Aste, M Giunti, P Famigli-Bergamini 339–342

Clinic and Ultrasonographic Findings in a Cat with Tetralogy of Fallot
A Fruganti, M Cerquetella, F Beribè, A Spaterna, B Tesei 343–346

Mitral Insufficiency in an Arctic Wolf
C Guglielmini, F Rocconi, L Brugnola, C Vantini, F Valerio 347–349

Personal Experiences in the Use of Association Tiletamine/Zolazepam for Anaesthesia of the Green Iguana (*Iguana iguana*)
M Mauthe von Degerfeld 351–353

Anticardiolipin Antibodies in Dogs
R Papini, O Pellegrini, C Rossini, G Guidi 355–357

Quantitation of the Cytokines TNF-α, IL-8 and IL-10 in Bovine Milk Using Real-Time TaqMan® PCR
A Peli, A Luciani, P De Santis, A Polci, D Britti 359–362

Case Report of Leishmaniasis in Four Cats
MG Pennisi, M Venza, S Reale, F Vitale, S Lo Giudice 363–366

Blood Pressure Measurements in Dogs and Horses Using the Oscillometric Technique: Personal Observations
F Porciello, F Birettoni, MB Conti, C Marinetti, MT Antognoni, G Fruganti 367–369

Swine Ochratoxicosis: Proteomic Investigation of Epatic Bioindicators
P Roncada, F Carta, M Zannotti, L Malagutti, F Sciaraffia, GF Greppi 371–375

Equine Cushing-like Syndrome: Diagnosis and Therapy in Two Cases
M Sgorbini, D Panzani, M Maccheroni, M Corazza 377–380

Veterinary Case History: Forensic and Medical Legal Aspects
G Signorini, G Biagi, E Luchetti, G Cardini, R Bernocchi 381–384

Serum Values of Cardiac Troponin-T in Normal and Cardiomyopathic Dogs
A Tarducci, O Abate, M Borgarelli, A Borrelli, R Zanatta, A Cagnasso 385–388

Postural Pattern Alterations in Orthopaedics and Neurological Canine Patients: Postural Evaluation and Postural Rehabilitation Techniques
C Vallani, C Carcano, G Piccolo, V Galardi, P Longhi, R Padovano, V Cantarini 389–391

Veterinary Research Communications, 28 (2004) 9–11

The role of Vet2020 Project on Quality of European Veterinary Education

T. Fernandes
EAEVE Chairman, Faculdade de Medicina Veterinaria, Universidade Tecnica de Lisboa, Lisboa, Portugal
Correspondence: Faculdade de Medicina Veterinaria, Universidade Tecnica de Lisboa, Rua Professor Cid dos Santos, Polo Universitario da Ajuda, 1300-477, Lisboa, Portugal
E-mail: titofernandes@fmv.utl.pt

BACKGROUND

The rapid pace of change in society's perception and the marking effects within the context of the Sorbonne–Bologna–Lisbon–Prague–Berlin process, has provoked an intensive debate on the nature of educational structures.

Educational structures do not aim at developing any sort of unified, prescriptive or definitive European *curriculum*, neither end the rich diversity of European educational systems, nor restrict the independence of academics, subject specialists or damage national academic autonomy.

Veterinary education has been, through the efforts of *EAEVE* – the *European Association of Establishments for Veterinary Education*, and several national Colleges/Orders/Councils, real pioneers in these events of comparative analysis, building upon their experience and conferring a European dimension to the undertaking by the means of a long established volunteer Evaluation System. Indeed, it was even granted an official mandate from the European Commission for this purpose. It must be stressed that it is not a European Accreditation System in the real sense, since the European Union (EU) do not assume such transnational scheme (having however specific legislation on Recognition of Professional and Academic Qualifications).

The professional profiles and learning outcomes were long defined as minimum requirements reference points and published by the European Commission (EC). Simultaneously, the EC has conducted a reformulation of old directives (dated 1978) in order to develop a model curricular structure for each sectorial area, including Veterinary Sciences. This has been up-dated by EAEVE in 2002 (www.eaeve.org/SOP).

A GLOBAL PERSPECTIVE ON CHANGE IN VETERINARY EDUCATION

Universities worldwide are facing numerous challenges including increasingly limited resource allocations, difficulties on enrollments, gender changes, keeping up with

advances in information and other technologies, remaining aware and responsive to users and the need to aggressively globalize their teaching, research and outreach programs.

We must learn to share resources, ideas and, most importantly, knowledge.

Generally speaking, people have yet to appreciate learning as a lifelong process. In many countries, economic difficulties are affecting or postponing the implementation of important measures to further the aims of lifelong learning (LLL). Furthermore, LLL will become a mainstream preoccupation for our faculties since it is essential for economic and social development.

The clear message is that traditional systems must be transformed to become much more open and flexible, so that learners can have individual learning pathways, suitable to their needs and interests, and thus genuinely take advantage of equal opportunities throughout their lives. Creating a culture of learning depends ultimately on increasing learning opportunities, raising participation levels and stimulating demand for learning.

Today, *curriculum* reform is an on-going process in most schools, increasing direct self-learning periods, reducing formal teaching, increasing practical and extra-mural services, introducing electives/optional oriented to animal species and future specialization areas.

Resources are declining significantly and this is likely to continue, meaning that one needs to be more imaginative and entrepreneurial in attracting the resources required to serve the public interest.

The formal system is still rigid and based on very old compulsory European Directives (EEC 1027/78, 1028/78) pointing to old-fashioned omni-competence. Despite many projects and programs initiated by the EU, and other bodies such as EAEVE, drawing up benchmarking for Veterinary Science, most *curricula* are not in modules, and there is little interplay between paths of learning.

CHANGES IN VETERINARY EDUCATION

The project VET2020 (www.fmv.utl.pt/vet2020), a market study on the future profile of a veterinarian according to society demands, that we have coordinated for the past 2 to 3 years among 22 European partner countries, has presented clear evidence that there are specific reasons why veterinary education needs reforming.

First of all, the agricultural/animal industry is declining in importance in most European countries while growing in developing countries, where population increases. Society in general, and gender of the veterinarian, is changing rapidly. The changing role of the veterinarian and the occurrence of new emerging diseases of animal origin and human implications, are strong reasons to justify a set of additional subjects or program adjustments. This could be the most important specific reason for changes in veterinary education, through its implications in Food Science, Animal/Public Health and Environmental Protection. There is a drastic need to

address food supply and security issues in the veterinary curriculum. Concerns about the environment and natural resource development must also be taken into consideration.

Nevertheless, despite the needs of common policies and legislation within the EU, it is very difficult to point out a model example to be copied by all schools in this process of change. One can learn from all processes and from all examples, but solutions have to be local. They must be studied and suited to local conditions, regularly updated, yet fulfilling the minimum requirements set up by specific guidelines/directives.

The process of change needs devoted and appropriate institutional management and leadership, teamwork, real definition of aims and goals, avoiding isolation, inbreeding, provincialism and inadequate quality control.

ACCREDITATION

There has been much discussion on various different fora concerning accreditation. This includes discussion with the European Commission concerning accreditation in the sense of the legal recognition of veterinary diplomas issued by establishments that meet a certain standard. The European Commission has explicitly stated that the current legal basis for recognition and free movement is that set out in the veterinary training directives. Any changes to the current system would require modification of EU law. Establishing a legally-based system of accreditation would require at least two major changes:

- The existing directives on veterinary training and the mutual recognition of veterinary diplomas/degrees would have to be revoked;
- A legal and professional framework would need to be established under new legislation whereby automatic recognition is given to diplomas delivered by accredited establishments, but whereby also further requirements can be imposed on individuals with diplomas from other establishments.

The European Commission has made it clear that it has no plans to embark on such changes without the political will of the Member States. The Commission has no competence to intervene in this way in higher education.

Veterinary Research Communications, 28 (2004) 13–28

Development of European Educational Strategies: Design of Veterinarian Profiles Identified by Market needs for the Year 2020

P. De Castro[1] and S. Zucconi*
[1] *Department of Veterinary Morphophysiology and Animal Production (DIMORFIPA), University of Bologna, Italy*
* *Correspondence: Nomisma SpA – Agricoltura e Industria Alimentare – Strada Maggiore 44 – 40125 Bologna, Italy*
E-mail: zucconis@nomisma.it

THE OBJECTIVES

The main aim of the VET2020 research project concerned the development of European educational strategies and the definition of the design of veterinarian profiles identified by market needs for the year 2020.

In particular, the VET2020 project aimed to identify:

1. Forecasts of the labour market demand for veterinarians through 2020.
2. Forecasts of the development of traditional professional activities through 2020.
3. Identification of new professional spheres through 2020.
4. Strengths and weaknesses of veterinary education and profession.

This project, undertaken under the auspices of research activity supported by the European Union's Socrates Programme, was coordinated by the Faculty of Veterinary Medicine of the Technical University of Lisbon. The survey was conducted by Nomisma, an Italian economic research institute. This research enjoyed the invaluable support and co-operation of the main European faculties of veterinary medicine.

The research activity involved 20 European countries: 13 EU Members (Austria, Belgium, Finland, France, Germany, Greece, Ireland, Italy, Netherlands, Portugal, Spain, Sweden, United Kingdom) – defined in this report as EU Countries – and seven countries that are not members of the European Union (Czech Republic, Hungary, Norway, Poland, Romania, Slovak Republic, Slovenia) – defined as Non EU Countries.

THE PROJECT

The VET2020 project was divided into two phases of research:

Phase A – Veterinary Profession Outlook

In this phase, a methodology was developed which permitted the defining of the most important indicators of the veterinary sector and established all the necessary elements with which to implement the successive phase of research.

Through careful bibliographic research and close collaboration with the main faculties of veterinary medicine of the 20 European countries involved in the survey, which supplied and validated the data making up this report, the following indicators have been defined:

1. The number of veterinarians and students.
2. The distribution of veterinarians according to fields of work.
3. The change in the number and gender of students over time.
4. The number of main livestock species.

The VET2020 project puts the number of veterinarians in these countries at 173,427; approximately three-quarters of these are found in the European Union (139,893) and the remainder reside in the Non EU Countries (33,534). These data were provided by the Faculties of Veterinary Medicine involved in the project (see Fig. 1).

Figure 2 illustrates the distribution of veterinarians by region.

For a better understanding of the importance of the veterinary profession in each of the countries involved in the survey, Table I shows the number of veterinarians per 1,000 inhabitants in each country. Four of the five most populous European countries – Germany, Italy, the United Kingdom and France – have values more or less similar, ranging from 0.309 per 1,000 inhabitants for France to 0.366 for Germany. Spain is

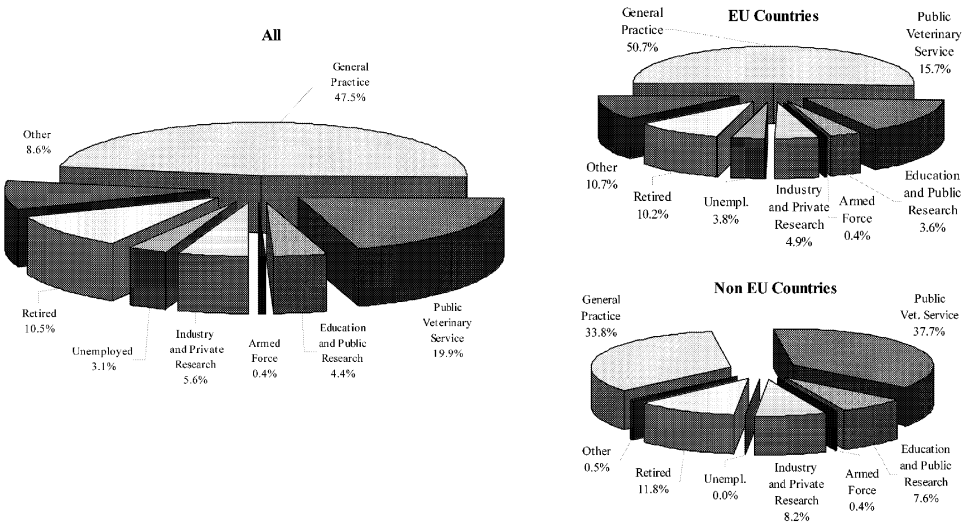


Figure 1. Veterinarians in Europe by Professional Field – 2001

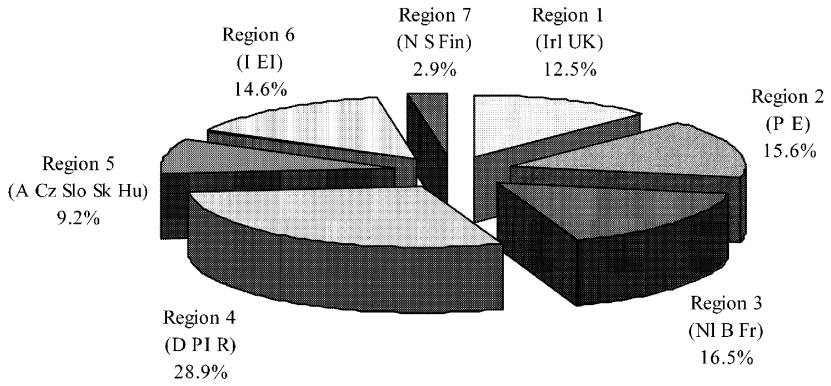


Figure 2. Veterinarians in Europe by Regions – 2001

TABLE I
Veterinary numbers/population ratio (per 1000 inhabitants) – 2001

EU Countries	Veterinary numbers/ population ratio	Non-EU Countries	Veterinary numbers/ population ratio
Spain	0.620	Slovak Republic	0.700
Belgium	0.569	Slovenia	0.651
Ireland	0.558	Norway	0.411
Greece	0.549	Hungary	0.389
Austria	0.382	Romania	0.409
Germany	0.366	Czech Republic	0.369
Italy	0.340	Poland	0.285
United Kingdom	0.329		
Portugal	0.324		
Netherlands	0.311		
France	0.309		
Finland	0.281		
Sweden	0.196		

Source: VET 2020 Preliminary Report.

the only big European country with a higher value, 0.620. The countries with the highest absolute values are the Slovak Republic (0.700) and Slovenia (0.651). Ireland, Greece and Belgium have values above 0.5. The countries with the lowest ratio of veterinarians are Sweden (0.196), Finland (0.281) and Poland (0.285) (see Table I).

In order to evaluate the prospects of the veterinary sector, it is opportune to verify not only the number of veterinarians, but also the number of veterinary students. The total number of veterinary students in the 20 countries covered in the survey is 55,469, 83.2% of whom reside in the EU; the remainder being from Non EU Countries. Here

it is also opportune to compare the number of veterinary students to the total population of each country under consideration. This is illustrated in Table II, where the data is presented as veterinary students per 1,000 inhabitants (see Table II).

For a complete analysis of the university system, Fig. 3 illustrates the total number of veterinary faculties present in each country.

Phase B – Implementation of the Survey VET2020

The results emerging from the analysis of Phase A, although carried out in an in-depth and critical manner, did not provide all of the elements necessary to gain an understanding of, for example, the demand for veterinary services in 2020, new fields of specialisation or the veterinary fields with the best employment prospects, nor did they help in establishing the strengths and weaknesses of the current system. The methodology of the survey was found to be the best method to compensate for this lack of specific information. This also allowed the successive phases of the project to be based on less extemporaneous knowledge.

So the second phase of the project entailed an original sample survey focusing on principals with direct experience in the veterinary field or those with a thorough knowledge of the main characteristics and changes affecting the future of the veterinary profession.

In particular, the following were carried out (numbers in brackets refer to completed questionnaires):

TABLE II
Veterinary students/population ratio (per 1000 inhabitants) – 2001

EU Countries	Veterinary students/ population ratio	Non-EU Countries	Veterinary students/ population ratio
Belgium	0.330	Slovenia	0.156
Austria	0.281	Slovak Republic	0.146
Spain	0.255	Romania	0.141
Italy	0.213	Poland	0.096
Portugal	0.189	Norway	0.077
Greece	0.134	Czech Republic	0.064
Ireland	0.105	Hungary	0.050
Finland	0.095		
Netherlands	0.089		
Germany	0.084		
United Kingdom	0.048		
Sweden	0.044		
France	0.034		

Source: VET 2020 Preliminary Report.

TABLE III
In your opinion, which are the strengths of the current university system in your country? (*Veterinarians Survey*)

	EU Countries		Non-EU Countries		All	
Countries	1st %	Multiple* %	1st %	Multiple* %	1st %	Multiple* %
Good basic education	53.0	60.1	29.2	35.9	48.3	55.3
Highly qualified teachers and staff	12.3	19.4	28.2	32.9	15.5	22.1
Good practical training	10.4	27.9	11.8	28.7	10.7	28.0
Modern equipment-structure of the Faculty	4.7	8.5	2.9	11.9	4.4	9.1
Small number of students	3.4	4.2	5.4	7.2	3.8	4.8
All veterinary fields are covered	2.6	7.8	4.0	6.1	2.9	7.4
Students selection	3.0	4.3	0.3	0.3	2.4	3.5
Specialized in some particular veterinary fields	2.8	5.4	3.5	6.4	3.0	5.6
Long education time	0.8	2.1	0.0	0.8	0.6	1.8
Cooperation with other universities	0.0	0.9	2.5	3.9	0.5	1.5
Other	7.0	23.2	12.2	25.8	7.9	23.7
Total	100.0		100.0		100.0	

*Multiple answers refer to the sum of responses given by respondents and indicate the proportion of respondents that gave a specific answer, independently of the order of priority. With reference to the above table for example, 60.1% of veterinarians in countries of the European Union, that consider the university systems in their respective countries to be very efficient, indicated a "good basic education" as a strong point of the system. This factor is indicated as the most important strong point by 53% (1st choice). In all of the following tables marked with an asterisk (*), multiple answers have been analyzed. The tables must be interpreted taking this into consideration.
Source: Nomisma VET2020 Survey.

1. Veterinarians Survey – VET Survey (823)

The VET Survey focused on veterinary professionals operating in different fields: practitioners, government veterinarians, professors or university researchers, veterinarians employed in industry.

2. VET Organisations and Employers of Veterinarians Survey – Employers Survey – (236)

For the Employers Survey, companies involved in the food sector, feed and pharmaceutical industries as well as veterinarian organisations were surveyed; these partici-

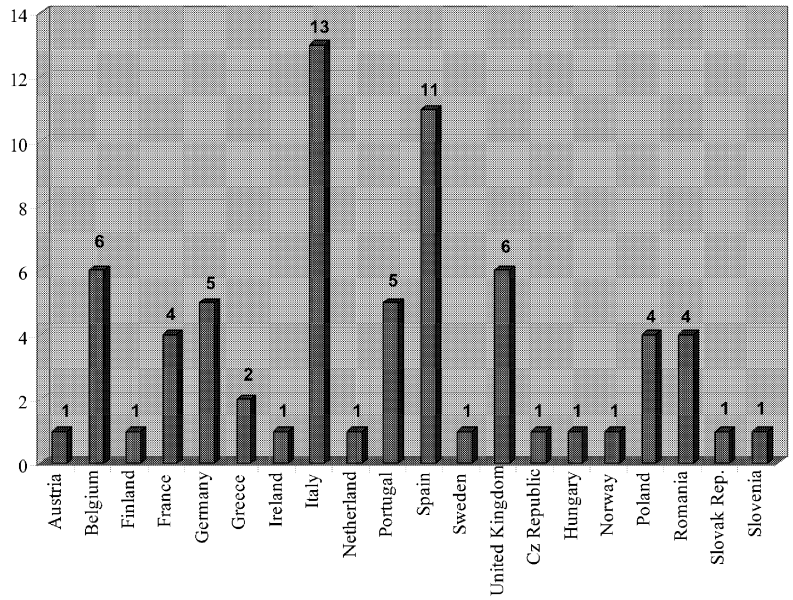


Figure 3. Faculties of Veterinary Medicine in Europe by Country – 2001

pants represented those who directly employ or can furnish employment to veterinarians.

3. Consumer Organisations Related to Veterinary Service Survey – Consumer Organisations Survey – (111)

The Consumer Organisations survey was aimed at associations whose members are habitual users of veterinary services.

For all three surveys, data was collected through questionnaires distributed by e-mail and was filled in by accessing a web page dedicated to the VET2020 survey (e.g., http://www2.nomisma.it:3110/Vet/index.htm). The survey was conducted according to the following process:

1. Initial contact – request for collaboration with VET2020 project by e-mail, with attached Survey presentation letter.
2. Reminder to non-respondents sent at regular intervals, every 10 days, up to a maximum of 5 solicitations.
3. For the fourth and fifth requests to respond, the country partner sent a personal request for collaboration to the non-respondents.

In general, response to the survey was very good. Contact by e-mail was a less intrusive way to conduct the survey, providing more freedom for the respondent to choose the best time in which to complete the questionnaire. This proved to be very

useful to the veterinarians and companies and organisations responding. Finally, using e-mail as the method for contacting respondents greatly reduced the time necessary for conducting the survey.

In order to understand the value of collecting data in this manner, it is important to point out that approximately 1,600 veterinarians were contacted for the VET Survey and 823 completed questionnaires were returned, giving a response rate of approximately 50%. A total of 3,600 e-mails were sent, including initial contacts and further requests for collaboration.

MAIN RESULTS

It was deemed important to evaluate perceptions of the efficiency of the current university system. The results obtained indicate a clear cut prevalence of positive evaluations and a high level of efficiency (see Fig. 4). 59.7% regarded the university system of veterinary education to be efficient or very efficient. This value is greater than 65% in Non EU Countries. 59% of the respondents in EU Countries had perceptions of efficiency. Differences appear in the analysis by region. In fact in some cases, the current university system is perceived better in respect to the evaluation given by the respondents of all the 20 countries involved. In particular, Region 1 (United Kingdom and Ireland), Region 3 (France, Belgium and Netherlands), Region 5 (Austria, Czech Republic, Slovenia, Slovak Republic and Hungary) and Region 7 (Finland, Norway and Sweden) demonstrate higher efficiency evaluations, while in Region 2 (Portugal and Spain), Region 4 (Germany, Poland and Romania) and Region 6 (Greece and Italy), the proportion of respondents that gave a not efficient

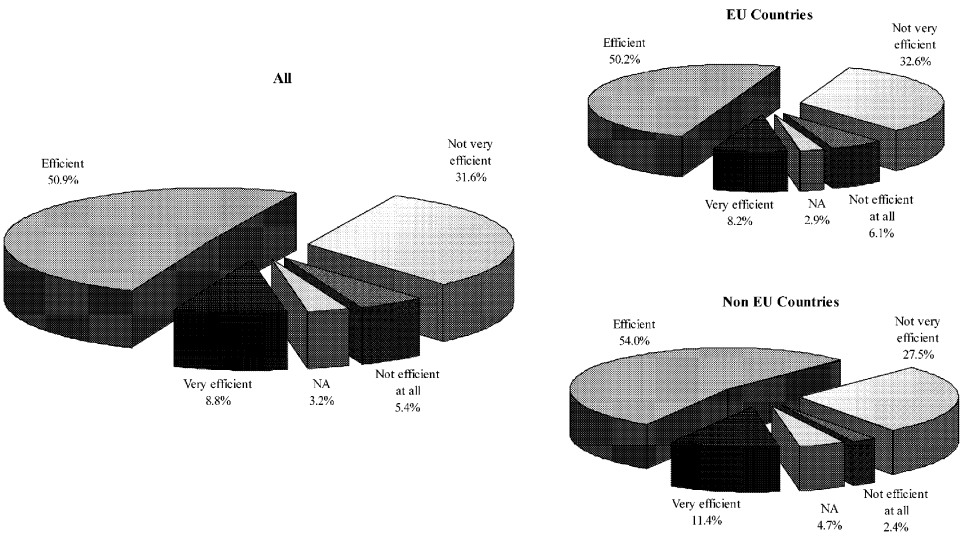


Figure 4. What is your evaluation of the efficiency of the veterinary training offered by the current university system in your country?

evaluation is superior to those who gave an efficient one. Furthermore, United Kingdom and Ireland (Region 1, 18.9%) and Finland, Sweden and Norway (Region 7, 16.2%) are distinguished by the highest evaluations, declaring that the university systems of their countries are "very efficient".

Tables III and IV permit a further evaluation, showing strong and weak points of the current university system.

Of all the respondents who perceived their institutions to be highly efficient, the most important point in the European university system was that of a "good basic education" guaranteed to students (48% as the first choice and 55% overall). Further

TABLE IV
In your opinion, why is the current university system so inefficient? (*Veterinarians Survey*)

	EU Countries		Non-EU Countries		All	
Countries	1st %	Multiple* %	1st %	Multiple* %	1st %	Multiple* %
Lack of clinical work/practice	24.2	46.3	54.5	67.9	30.2	50.6
Too many students	17.6	21.6	1.7	1.6	14.5	17.7
Education is far from the market needs	15.8	26.1	3.1	8.9	13.3	22.7
Poor professionalism of teachers	10.0	19.6	16.0	11.7	11.2	18.0
Lack of funds by government for research	4.2	6.3	4.7	6.0	4.3	6.2
Too much specialization	4.7	5.8	0.0	2.5	3.7	5.2
Too many academics	4.2	4.4	0.0	0.0	3.4	3.5
Lack of specialization	2.6	7.3	5.0	6.4	3.0	7.1
Not enough flexibility	3.0	3.4	1.7	2.1	2.7	3.1
Lack of knowledge in other areas (e.g. marketing, economics, etc.)	1.6	4.8	1.7	5.2	1.6	4.9
Old equipment	0.8	5.5	0.0	26.0	0.7	9.6
Too many VET faculties	0.0	2.3	0.0	0.0	0.0	1.9
Other	11.3	16.6	11.6	21.0	11.4	17.5
Total	100.0		100.0		100.0	

*Multiple answers refer to the sum of responses given by respondents and indicate the proportion of respondents that gave a specific answer, independently of the order of priority. With reference to the above table for example, 60.1% of veterinarians in countries of the European Union, that consider the university systems in their respective countries to be very efficient, indicated a "good basic education" as a strong point of the system. This factor is indicated as the most important strong point by 53% (1st choice). In all of the following tables marked with an asterisk (*), multiple answers have been analyzed. The tables must be interpreted taking this into consideration.

Source: Nomisma VET2020 Survey.

elements of efficiency which were well appreciated are "highly qualified teachers" (15.5% and 22.1% respectively) and "good practical training staff" (10.7% and 28%).

When comparisons are made between EU and Non EU Countries, no real differences emerge, with the exception of the importance that Non EU Countries veterinarians assign to the "qualification of teachers and staff". Also, the method for "student selection" appears to be more important for EU countries.

With regard to weak points, the responses seem to be concentrated on a limited number of factors. The lack of clinical experience is considered by far as the most important weak point (30.2% and 50.6%) at a group level and this is even more pronounced in the Non EU Countries (54.5% and 67.9%). Excessive numbers of students (17.6% and 21.6%) and the lack of coherence in educational programs to meet market demands (15.8% and 26.1%) appear to be quite problematic, particularly in countries of the European Union. Common to both groups is the perception of a poor professionalism of teachers in the teaching staff.

The shared opinions on weak and strong points permit a better understanding of information relative to modality and tools for improving the educational training offered by veterinary faculties in the European university system (Table V).

By far the most important factor cited (22% of first choices and 39% overall) was a deeper practical and clinical experience. This was even more strongly suggested by veterinarians from the Non EU Countries. Following this, in descending order of importance are a limited number of students (above all in EU countries), more availability of funds (Non EU Countries), teaching motivation by professors and more cooperation with the private sector (EU countries). In addition to adjustments in the European university system, it was further requested to develop the tools necessary to guarantee to the students a level of competence capable of meeting the challenges and demands of the next twenty years. Another important objective of the VET2020 Survey was to identify, according to the different categories of respondents, the opinion on the changes to be seen in 2020, in the number of veterinarians employed in the respective countries. The survey enables us to estimate the number of working veterinarians in 2020.

The first important finding, confirmed by all three surveys, regards the increase in the number of veterinarians employed in 2020. 58.3% of the responses received from all the 20 countries covered in the VET Survey indicated that they expected growth in the number of employed veterinarians in 2020.

Analogous predictions emerged in the other two surveys: 76.1% of respondents to the Employers Survey and 54.1% of the participants in the Consumer Organisations Survey in the 20 countries indicated growth as well.

Growth predictions are confirmed for both the European Union and Non EU Countries, with some differences in intensity, as can be seen in the following table. The balance represents the difference between those who indicated an increase and those who predicted a decrease, net the proportion of those who foresaw no change at all.

The prediction of an increase in professional opportunities for veterinarians in 2020

TABLE V
How could the current veterinary schools in your country be improved? (*Veterinarians Survey*)

	EU Countries		Non-EU Countries		All	
Countries	1st %	Multiple* %	1st %	Multiple* %	1st %	Multiple* %
Deeper practical and clinical experience	20.1	35.4	31.7	52.2	22.4	38.7
Less students	12.1	15.5	6.6	11.3	11.0	14.7
More funds for education and research	5.2	7.4	9.7	13.9	6.1	8.7
Well motivated teachers	6.6	14.0	1.7	4.1	5.6	12.1
Cooperation between university and industry	6.2	15.6	1.7	3.8	5.3	13.2
Review of the post-graduate education system	5.8	11.2	2.3	7.0	5.1	10.4
Improving the working conditions of the teachers	5.6	12.6	1.3	4.3	4.8	11.0
A new curriculum/different study plan	4.4	12.3	3.0	5.0	4.1	10.9
Competence in other areas (e.g. marketing, economy, etc.)	2.5	7.9	1.6	10.8	3.2	8.5
More cooperation with other faculties	2.6	5.2	2.7	9.9	2.6	6.2
More attention to market needs	2.2	6.2	1.6	3.6	2.1	5.7
Longer/broader basic education	2.2	3.1	0.0	0.2	1.8	2.5
Modern equipment and infrastructure	1.3	7.3	3.1	15.4	1.6	8.9
Less vet faculties	1.6	6.4	0.4	0.5	1.4	5.2
Introducing a new teaching method	1.1	3.6	1.9	7.3	1.3	4.3
Other	8.9	25.3	9.6	21.4	9.0	24.6
No Answer	10.6		21.1		12.6	
Total	100.0		100.0		100.0	

*Multiple answers refer to the sum of responses given by respondents and indicate the proportion of respondents that gave a specific answer, independently of the order of priority. With reference to the above table for example, 60.1% of veterinarians in countries of the European Union, that consider the university systems in their respective countries to be very efficient, indicated a "good basic education" as a strong point of the system. This factor is indicated as the most important strong point by 53% (1st choice). In all of the following tables marked with an asterisk (*), multiple answers have been analyzed. The tables must be interpreted taking this into consideration.
Source: Nomisma VET2020 Survey.

with respect to the present constitutes an important result of the survey, confirmed not only in the different geographical areas but also reinforced by the expectations of those who utilise professional veterinary services (see Table VI).

In the VET Survey it was possible to verify the intensity of forecasted levels of growth in various areas of the continent. The following figure shows the balance in the seven identified regions.

To summarize the opinions expressed by individual regions, Fig. 5 proposes, for each region, a "Balance Value", the value that represents the difference between those predicting an increase in the number of working veterinarians and those foreseeing a decrease, net of the proportion of respondents that expressed the opinion that the number of working veterinarians would remain stable. It is possible to immediately note that for all the regions the balance value is positive. This signifies that the prevalent prediction of growth in the number of employed veterinarians is transversal and shared by all the Regions. It is easy to verify how the results of the entire survey (Total) are strongly influenced not only by Region 7 (Sweden, Norway and Finland), but also by Region 3 (France, Belgium and Netherlands) as well as countries of Region 2 (Spain and Portugal), all more important in numerical terms in relation to the number of veterinarians present. All other areas appear to be under the total European value and in particular, the estimates of growth appear especially low in Region 2 (9.3% of balance value), in Region 1 (United Kingdom and Ireland, 12.4%) and Region 4 (Germany, Romania and Poland, 10.7%). To better understand the directions of such growth and spell out the implications for training and educational requirements, the investigation conducted an in-depth analysis into the opportunities Non EU with individual professional fields of veterinary medicine.

TABLE VI
The estimated change in the number of employed veterinarians in 2020 in the country of the respondents – *a comparison of the three surveys*

	EU Countries			Non-EU Countries			Total Countries		
	VET	EMPL %	CONS ORG	VET	EMPL %	CONS ORG	VET	EMPL %	CONS ORG
No change	8.0	8.1	15.4	5.9	14.8	30.2	7.6	9.2	18.1
Moderate increase	40.5	64.4	45.0	42.7	47.1	46.2	41.0	61.6	45.2
Sharp increase	19.2	13.9	10.9	9.8	17.6	0.0	17.3	14.5	8.9
Moderate decrease	19.4	8.9	18.3	25.4	14.8	23.3	20.6	9.8	19.2
Sharp decrease	9.7	1.1	6.9	7.6	3.4	0.3	9.3	1.5	5.7
No Answer	3.2	3.6	3.5	8.6	2.3	0.0	4.2	3.4	2.9
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Balance	30.7	68.3	30.7	19.4	46.4	22.6	28.5	64.8	29.2

Source: Nomisma VET2020 Survey.

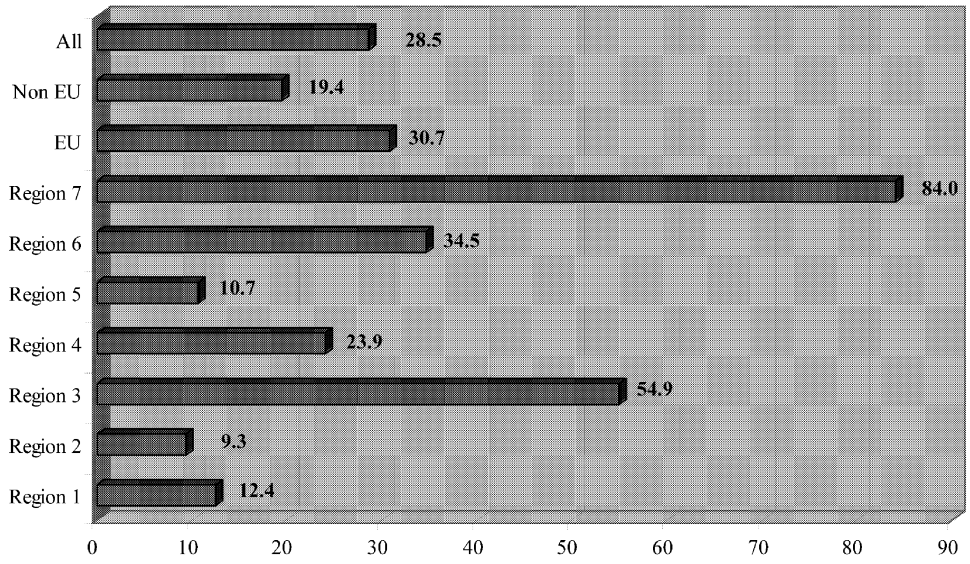


Figure 5. Regions: The estimated change in the number of employed veterinarians in 2020 in the country of the respondent: balance

The following three tables synthesize the results of the forecasts referring to veterinarians employed in different fields of activity in 2020. The forecasts for veterinary employment in each field were formulated taking into consideration the prevalence of the responses furnished by the respondents. They are listed in decreasing order in terms of the percentages of respondents who, respectively, expected growth, stability and decrease in job opportunities. Furthermore, the diagrams illustrate the differences between results in European Union Countries and the Non EU Countries.

All three groups surveyed shared the opinion that the number of veterinarians who will find work in the field of "Food Quality and Safety" in 2020 will increase. There are, however, some differences at a geographical level in terms of the growth expectations for Other fields of professional activity, which can be identified by observing the preceding diagrams. Furthermore, it should be noted that among the professional fields where a decrease in job opportunities has been predicted over the long-term, "Experimentation on Animals" was prevalent (only the Employers predicted stability in the number of jobs in this field). Although having different proportions, analogous situations seem to be present in the different geographical areas taken into consideration.

Figure 5 shows the most important fields in terms of growth, stability or decrease for each of the seven regions. The trend for each geographical area was identified by the prevalence of the responses by veterinarians of each region. Only the field "Food Quality and Safety" appears among the first three choices for every region and seems to be the most highly considered element in Region 1 (Ireland and United Kingdom),

TABLE VII
According to your estimate, what will be the change in the number of employed veterinarians in 2020 in your country in the following fields? (*Veterinarians Survey*)

EU Countries	Non EU Countries	Total
INCREASE		
Food Quality & Safety	Pet/Companion Animal	Food Quality & Safety
Exotic Animal	Food Quality & Safety	Public Health
Public Health	Environmental Protection	Exotic Animal
Alternative Medicine	Animal Welfare	Environmental Protection
Environmental Protection	Sport Animal	Alternative Medicine
Animal Welfare	Public Health	Animal Welfare
Pet/Companion Animal	Exotic Animal	Pet/Companion Animal
Epidemiology	Alternative Medicine	Epidemiology
Organic Farming		Organic Farming
		Research & Development
STEADY		
Sport Animal	Epidemiology	Sport Animal
Research & Development	Aquaculture/Fish Farming	Aquaculture/Fish Farming
Aquaculture/Fish Farming	Research & Development	
	Animal Breeding	
	Organic Farming	
DECREASE		
Experimentation on animal	Herd Health	Experimentation on animal
Herd Health	Experimentation on animal	Herd Health
Animal Breeding		Animal Breeding

Source: Nomisma VET2020 Survey.

Region 2 (Portugal and Spain), Region 3 (Netherlands, France and Belgium) and Region 7 (Finland, Norway and Sweden). In Regions 4 (Germany, Poland and Romania) and Region 6 (Greece and Italy) the future role of "Pet and Companion Animals" seems to prevail. Region 5 (Austria, Czech Republic, Hungary, Slovak Republic and Slovenia) showed a prevalence of respondents indicating activity regarding "Environmental Protection." In the same way, the prospects appear rather homogeneous in respect to the fields identified as decreasing.

TABLE VIII
According to your estimate, what will be the change in the number of employed veterinarians in 2020 in your country in the following fields? (*Employers Survey*)

EU Countries	Non EU Countries	Total
INCREASE		
Food Quality & Safety	Food Quality & Safety	Food Quality & Safety
Pet/Companion Animals	Environmental Protection	Environmental Protection
Alternative Medicine	Animal Welfare	Pet/Companion Animals
Environmental Protection	Public Health	Public Health
Public Health	Organic Farming	Animal Welfare
Animal Welfare	Pet/Companion Animals	Alternative Medicine
Exotic Animals	Alternative Medicine	Exotic Animals
Organic Farming	Herd Health	Organic Farming
	Epidemiology	Epidemiology
	Research & Development	Herd Health
	Exotic Animal	
STEADY		
Research & Development	Aquaculture/Fish Farming	Research & Development
Animal Breeding	Experimentation on animals	Sport Animals
Sport Animals	Animal Breeding	Aquaculture/Fish Farming
Experimentation on animals	Sport Animals	Animal Breeding
Aquaculture/Fish Farming	Exotic Animals	Experimentation on animals
DECREASE		
Herd Health		

Source: Nomisma VET2020 Survey.

TABLE IX
According to your estimate, what will be the change in the number of employed veterinarians in 2020 in your country in the following fields? (*Consumer Organizations Survey*)

EU Countries	Non EU Countries	Total
INCREASE		
Food Quality & Safety	Environmental Protection	Food Quality & Safety
Public Health	Animal Welfare	Public Health
Animal Welfare	Food Quality & Safety	Animal Welfare
Alternative Medicine	Public Health	Environmental Protection
Pet/Companion Animal	Organic Farming	Pet/Companion Animal
Environmental Protection	Herd Health	Alternative Medicine
Epidemiology	Pet/Companion Animal	Herd Health
Herd Health	Sport Animal	Epidemiology
Research & Development	Epidemiology	Organic Farming
Organic Farming	Research & Development	Research & Development
Exotic Animal	Alternative Medicine	Sport Animal
STEADY		
Animal Breeding	Aquaculture/Fish Farming	Animal Breeding
Sport Animal	Experimentation on animal	Exotic Animal
	Animal Breeding	
	Exotic Animal	
DECREASE		
Experimentation on animal		Experimentation on animal

Source: Nomisma VET2020 Survey.

TABLE X
Regions: According to your estimate, what will be the change in the number of employed veterinarians in 2020 in your country in the following fields? (*Veterinarians Survey*)

	Increase	Steady	Decrease
REGION 1	Food Quality & Safety Public Health Exotic Animal	Aquaculture/Fish Farming Sport Animal Research & Development	Herd Health Experimentation on Animal Animal Breeding
REGION 2	Food Quality & Safety Public Health Animal Welfare	Sport Animal Research & Development Aquaculture/Fish Farming	Experimentation on Animal
REGION 3	Food Quality & Safety Public Health Exotic Animal	Sport Animal Research & Development Aquaculture/Fish Farming	Herd Health Experimentation on Animal Animal breeding
REGION 4	Pet/Companion Animal Environmental Protection Food Quality & Safety	Epidemiology Animal breeding Public Health	Experimentation on Animal Herd Health
REGION 5	Environmental Protection Animal Welfare Food Quality & Safety	Epidemiology Public Health Research & Development	Animal breeding Experimentation on Animal Herd Health
REGION 6	Pet/Companion Animal Aquaculture/Fish Farming Food Quality & Safety	Research & Development Environmental Protection Organic Farming	
REGION 7	Food Quality & Safety Public Health Pet/Companion Animal	Sport Animal Exotic Animal Animal breeding	Herd Health Experimentation on Animal

Region 1 (UK, Irl); Region 2 (E, P); Region 3 (B, Fr, Nl); Region 4 (D, Pl, Ro); Region 5 (A, Cz, Hu, Slo, SK); Region 6 (I, El); Region 7 (S, N, Fin).

Source: Nomisma VET2020 Survey.

Veterinary Research Communications, 28 (2004) 29–44

Quality Assurance: The Key for Amendments of the EU-Directive/s Regulating Veterinary Training in Europe

H. Rodríguez-Martínez
Associate Dean, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences (SLU), Department of Obstetrics and Gynaecology, Box 7039, SE-750 07 Uppsala, Sweden
E-mail: heriberto.rodriguez@og.slu.se

SUMMARY

The free movement of persons, goods and services within the European Union (EU) is one of the major principles established by the European treaties. This free movement shall now be reinforced through the full application of the new general system for the mutual recognition of professional qualifications, in which veterinary medicine is included. The success of this measure for internal market development imposes availability of professionals with the highest possible basic training and opportunities for continuing education and specialisation. Such benchmark definition requires the establishment of veterinary training throughout the EU to focus on the qualitative aspects of the basic training they impart. New production forms, new labour markets and a higher degree of consciousness of the producers and the consumers, together with an ever-increasing load of new information and knowledge in most veterinary fields had forced changes in veterinary education strategies. These changes have led to the adaptation of curricula and the application of new pedagogical concepts ultimately leading to the design of new, exciting programmes of veterinary training. Some of them use a combination of basic education and elective terms while others have focused training in species-oriented tracks already by the time students enter the clinical level. There is general consent that the quality of basal training must enable the student to achieve a level of confidence in life-long learning so he/she would be able to follow relevant CPD's and, eventually, pursue specialisation. At the same time, veterinary establishments are concerned with their ability to achieve these goals, mostly due to the usual high costs of veterinary training that constrain their chances to maintain equality of training levels through the EU. We need to find tools to harmonise veterinary training among the establishments of veterinary education in Europe, beyond the compulsory subject and training minimum requirements laid down by the Directive 78/1027. Harmonisation requires regulations but also awareness. Establishments of veterinary education must not only comply with regulations but also become aware of the advantages of quality assurance of their basic training. The present paper is a series of personal reflections by the author who ultimately addresses veterinary educators and interest organisations such as the European

Association of Establishments for Veterinary Education (EAEVE) and the Federation of Veterinarians of Europe (FVE) to focus on strategies of quality assurance as the basis for claims of amendments of the EU-Directive/s regulating veterinary training in Europe.

INTRODUCTION

Undergraduate veterinary education shall lead to formal qualification to pursue the activities of a veterinarian. The classical view is that a veterinary professional is to be in his core a well-trained clinician, able to take care of diseased animals. However, the professional field for a veterinarian is widening. Two major factors can be identified: the even-increasing bulk of knowledge in veterinary medicine and, perhaps more importantly, the request from society that the veterinary profession undertakes roles relevant to the re-assurance of human well-being, in terms of public health, but also regarding the increasing consciousness in issues of animal welfare, sustainable animal production and environmental protection.

In 1999/2000, an EU-financed project (VET2020; Development of European Educational Strategies: Design of Veterinarian Profiles Identified by Market Needs for the Year 2020, SOKRATES Thematic Network project 10042-CP-1-(99)2000-1-PT-ERASMUS-ETN), hereby named "Vet2020" was launched, whose aim was to explore the market needs for different profiles of veterinarians for the year 2020. The project ultimate aim was to use these data to signal ways for further development and, eventually, the harmonisation of the veterinary curricula among the European establishments of veterinary education.

As a major component of the project, a survey was conducted by a sub-contractor (Nomisma Spp, Italy), to determine the presence of trends indicative of different market needs for some veterinary profiles. The survey attempted to gather the opinion of a randomised sample of professionals, in as many different working fields as possible, to express their opinion on curricular issues and areas of expected development by 2020. Other important actors were also included such as employers of veterinarians, consumers of veterinary services, professional associations, etc. The results, although emanating from an overall rather small sample which might be considered by some countries/regions as insufficient or of low reliability, yield a series of interesting trends of opinions whose further analysis has been presented at the 5th General Meeting of VET2020 held in Toulouse on May 24, 2003 (Seminarium on "Future directions for veterinary education"). The analysis of the data emanating from the survey shall constitute the basis for the Final Report of the VET2020 project, to be ready for deliverance to the Commission by the project Co-ordinator following the 6th General Meeting (Lisbon, September 20, 2003). From the Nomisma report, the overall opinion (by all target groups surveyed) signalled the veterinary profession is not expected to decrease its present level of engagement among European professions, neither in terms of needs (employers and consumer organisations) nor

expectations (consumer organisations, professional organisations, professionals, etc.). Professionals regarded veterinary education as dynamic, and could foresee – according to the survey – changes in the curricula that better adapt to expected needs of the market. Among these, the survey identified the continuation of a good, yet perhaps diversified and much more specialised clinical training; as well as an increase in the training of particular issues of utmost importance for the protection of consumers' health, such as food quality and safety or veterinary public health. Among the employers and, particularly, the consumers' associations (the ones the market listen carefully to), areas such as veterinary public health, including herd-health and surveillance were among those predicted to harbour the highest opportunity for employment by 2020. As a whole, even noting the presence of regional and national differences, the survey clearly marked the need for adaptations of the curricula in veterinary training so that they best convey to the expectations of the responders, eventually leading to a stronger harmonisation among the establishments of veterinary education in Europe.

The present paper is a series of personal reflections by the author who ultimately addresses veterinary educators and interest organisations such as EAEVE and FVE to focus on strategies of quality assurance as the basis for claims of amendments of the EU-Directive/s regulating veterinary training in Europe. The paper includes the rationale behind initiatives undertaken by the VET2020 Steering Committee, in this respect.

VETERINARY UNDERGRADUATE EDUCATION: WHERE ARE WE HEADING FOR?

Veterinary undergraduate education was established in Europe about 250 years ago, as an answer to the needs of sectors of the society that needed skilful craftsmen, able to cope with the diseases of the moment. Noteworthy is to remember that veterinarians were, from the beginning, engaged in the establishment of preventive measures for the animal plagues that were a threat for the animal population and the humans that either consumed their products, cohabited with them, or both. From being initially directed towards the equine species for its role in transportation, haulage and for their role in less noble causes for mankind (warfare), more attention was later paid to buiatrics, due to the recurring epizootics among cattle, followed by other food-producing animals.

Veterinary education establishments, the educators that shape the impact of these establishments, and the veterinary profession as a whole have a lot to be proud of since two and a half centuries ago. The veterinary profession continues to play leading roles in alleviating the suffering of animals kept for recreational, social or production reasons, on the control of zoonotic diseases, as well as in increasing the animal health status of food-producing animals to such an extent that their potential productivity of human foodstuff has in many countries reached top levels and contributes to better

human health. Veterinarians are also taking part in the development of science, often at the very cutting edge, in areas involving genetics, microbiology, nutrition and reproduction. We continue to produce highly qualified and talented graduates who contribute to society in many different ways. It is, therefore, our duty to see that veterinary undergraduate education continues to develop to ensure the best possible training of academic professionals. In that duty is included our need to face changes with a positive attitude, making the necessary critical analyses considering our strengths and weaknesses and formulating imaginative plans to meet these challenges.

Educators have always faced the increasing volume of scientific knowledge and formulated their teaching strategy accordingly. Today we also face the increasing expectations of a public (as producers, consumers or decision-makers) that in a more critical manner, often related to the higher flow of popular information, questions the quality of the veterinary profession. The above factors lead to an increasing interest of the professionals to pursue specialisation after graduation.

Educational establishments are confronted by two major needs: new curricula of veterinary education that can deliver academic professionals trained for a life-long learning as well as the training of new educators and postgraduates that would ensure the function and future development of veterinary education and research. Obviously, these two needs become tasks which can only be tackled if the quality of our function as training institutions is high, and sustainable.

DEVELOPMENT OF VETERINARY EDUCATION IN EUROPE

Veterinary undergraduate education in Europe (and perhaps worldwide) is probably still considered one of the most traditional university educations, with extensive formal teaching, based upon rigid curricula, most often teaching facts rather than principles. Another characteristic, owing to its legal professional status, is the in-built requirement for a ground of basic training that should be broad enough to warrant the graduate to practise, in principle, in any field of veterinary medicine. Such equation, of getting qualified training in every field within a limited timeframe, is not solvable. Possible solutions have been, and are being, tested among establishments that recognise this dilemma.

In most places throughout Europe, efforts are now made to train students to develop basic skills but also to make them aware of the urgent need to seek information and to learn how to learn to make use of the information gathered. They should be, one would hope, better trained in problem-identification, problem-solving and decision-making. Basically, educators nowadays aim to engage veterinary education in the worldwide trend of life-long learning of our students instead of allowing them to memorise facts in order to pass examinations at the end of their courses.

As well, throughout Europe, there is an increasing awareness for implementing pedagogical approaches such as problem-based learning (PBL)-strategies in the pre-

clinical years in order to train the students to study in a different manner than what has been praxis until now. An early exposure to veterinary working fields, to the clinical subjects and a larger degree of interaction between educators from different institutions are also among the implemented strategies. Inclusion of new subjects, attempting the elimination of the sacred limits between disciplines are now more common, and we use more extramural training and elective courses or activities to be able to cope with the requirements of society. Accompanied with proper didactics, the clinical subjects have evolved from treating individuals to think more globally, involving societal needs, animal welfare, economics and epidemiological aspects. Classical subjects, such as food hygiene, are now widened into Veterinary Public Health for instance, and population medicine, herd health and surveillance are now developing away from the classical clinics of individual food-producing animals.

IS THERE A COMMON STRATEGY FOR CURRICULUM DEVELOPMENT?

More and more establishments of veterinary education assume that there is major need for defining a core curriculum; either because of the inherent claim that all graduates should receive the same legal status to practise the profession, because it contributes to the integration of course components and also because it aids the development of elective periods, diversification or even the adoption of restricted individual programmes. Defining a core curriculum provides larger benefits when a PBL-learning strategy has been applied at the beginning of the veterinary programme. Problem-based learning involves the use of clinical problems (sometimes cases, but these can very well be of a population nature rather than individual) in order to create an active, student-centred learning environment. Considering that most veterinary problems often require an interdisciplinary approach, students learn how to study basic and clinical sciences in an integrated fashion. This training of how to learn should be easily made permanent by the student, since it does not have to be maintained in the programme scheme along the curriculum, but rather be applied by the students themselves after passing the pre-clinical period. Particular advantages of an early application of PBL-strategies are the self-direction of learning, obtaining interdisciplinary knowledge, as well as the development of personal skills, including personal communication skills. Both analytical and technical/clinical skills need to accompany this approach of learning, particularly with proper hands-on training. However, educators must be convinced that the PBL-strategy is not just another fancy method of instruction but a new philosophy of education, focusing on the students' own work, so that they can grow as independent learners.

Veterinary knowledge increases, as well as the flow of information rising beyond our capability of pressing it within a formal curriculum of 5 or 5.5 years of instruction. The development of new technologies in many para-clinical and clinical subjects (including artificial reproduction technologies, specialised surgical sub-disciplines, imaging technologies, etc.) in the undergraduate syllabus continues to grow. There is

a society-driven increment in the diversity of species which veterinarians working as clinicians are to be consulted about, and farm animal clinics evolve towards preventive medicine, including issues of herd-health surveillance and risk assessment rather than the classical handling of individual illnesses. All these changes diminish our chances of reducing the already overcrowded veterinary curriculum.

Counter-measures are already being tested, some of them successfully. Provision of electives and/or tracks can individualise the programmes of veterinary undergraduate education. Elective periods, often at the end of a common part of the curriculum and lasting a few weeks to 1 or even 2 semesters, help the students to identify an area of particular interest and deepen into it. The area can include courses (either of narrow or broad nature) or activities (primarily skill-training in a particular subject or series of subjects). In some cases, elective periods are associated (with advantage) to the task of pursuing a project work, also in a subject the student selected by her/himself. The introduction of elective periods is a good measure aiming at reducing the omni-competence we have discussed above, but it has shown deviations (add-ons) and it does not necessarily limit the core per se. Tracking implies a new type of curriculum design, where the student opts to emphasise a specific area of veterinary medicine, so that a considerably enhanced level of knowledge is achieved at graduation. Even not being a specialisation (a level that would be obtained after graduation) tracking takes the student to a more useful level of competence in that particular chosen field, limiting redundant information and facilitating a more focused learning. Its major argument is that it limits information overload in the curriculum. However, tracking is not common yet, and many concerns have been raised in establishments that have opted to consider this option. Firstly, it complicates an already rather complex veterinary curriculum and forces the decision by young students (maturity is not an issue to be discussed here, but it has been named) to opt for a track, sometimes rather early in the curriculum. Tracking is at the very end a decision to be made by a certain establishment in the light of the socio-economic values and needs for veterinary professionals. In some areas (as is the case of University of California at Davis, USA) the practising veterinarian has clearly evolved from an omni-competent, primary healthcare provider for several species, to a single species health specialist, a disciplinary specialist or even a species-specific discipline specialist. However, there are many regions of the world where the predominant need still is for omni-competent, multi-species veterinarians.

Tracking or path-differentiation will no doubt be discussed further and some establishments in The Netherlands, Belgium, Italy and the Nordic countries are already approaching a more differentiated form of curriculum. In Utrecht for instance, the new curriculum (evolved from the study plan from 1995, and initiated 2001) includes separate educational paths (individual animals, farm animals, veterinary public health, veterinary scientific research and veterinary administration and management), enrolling on a 6-year long curriculum that attempts an increasing species-differentiation in practice as major output.

IS CURRICULUM DEVELOPMENT PROBLEM-FREE?

Changes in curricula among veterinary schools in Europe have improved training. Many students are more prone towards academic training, more interested in developing communicative skills, and are also better skilled in problem-solving. However, not all students see the necessity for improving communication skills, nor do they see the need of accomplishing academic skilfulness, since they want to become a veterinarian as fast as possible. Development of problem-solving skills is often seen as a slow process that goes against the students' inbuilt interest in gathering as many study credits as fast as possible. Last but not least, most students want to know and do all within the veterinary field.

Is this a student-restricted problem? Not really. Educators are a part of the problem. Students find it comfortable and safe to receive information via theoretical lectures where the educator tries her/his best to cover everything in the subject, with the false conviction that providing all information is the right thing to do. With such a conviction, the educator is reluctant to reduce the content of theoretical classes, and ultimately, of their own discipline in the curriculum, unless they are involved in multidisciplinary teaching. Although already repeated in as many opportunities as provided by conferences and courses at national, regional and even at EAEVE's level, the ideal of full coverage is still a major component of the problem. Students must at any given course be provided with tools for seeking knowledge and further information than what is given in the syllabus. Not least important, students must have enough time to convert information into knowledge.

In less and less schools throughout Europe, but still in too many, the number of students is too large. There must be a certain educator:student ratio in order to be able to provide the best possible training. The larger the number of students related to the number of teachers the worst would happen; lectures will be the consequence, and demonstrations will replace hands-on practicals … Furthermore, low educator numbers are usually connected to few academic staff numbers per institution, with the logical consequence that these are tied to teaching hours, and where any curricular change, even of a small nature, is seen as a threat and a burden impairing other relevant University activities such as research and research education, for instance. Sometimes this matter must be handled in two directions, firstly, assuming that *numerus clausus* must be discussed and accepted as a need and, secondly, understanding that sometimes an academic structure based on larger departments is to be preferred.

Are these scenarios working against core curricula? Yes, having teachers (and professionals) with such a conservative view is translated into students feeling they are not provided with the broadest possible training, which they assume must be the best. In establishments where core curricula have been imposed in a top-down approach, educators often responded with a document (often very, very large) of a core syllabus, where almost everything is listed for the student to understand she/he

must know a bit of everything, so that treating "all creatures large and small" can be warranted ... This misunderstanding simply contributes to maintain the bad circle.

Educators are themselves, as a whole, not happy. Frustration is not infrequent, especially for those subjects that would be considered more "academic" than "craftsmanship-oriented". A visible consequence among veterinary establishments worldwide is the worrying lowering number of veterinary graduates enrolling in postgraduate veterinary training. Whether this is caused by low salaries among university staff members compared to professional positions is always a matter of discussion, but a fact is that academic positions in the pre- and para-clinical departments are being filled by scientists without veterinary degrees, most often against the clear strategic wishes of the institutions.

The major problem we are facing nowadays is the disparity, still present after so many years of genuine work with curriculum development, among establishments of veterinary training in the EU.

QUALITY OF BASIC VETERINARY TRAINING IN EUROPE: REGULATIONS AND REQUIREMENTS

The free movement of persons, goods and services within the European Union (EU) is amongst the major principles established by the European treaties. The veterinary profession is fully included in this movement through the automatic recognition of the professional diplomas granted by the EU establishments of veterinary education (Council directives 89/48/EEC and 78/1026/EEC). This recognition relies on the assumption that an equivalent level of veterinary training is provided throughout, as required by the Directive 78/1027 (Council Directive 78/1027/EEC), which establishes a minimum of subjects and overall timeframes to be included in the veterinary curriculum.

Both Directives enshrined into Community law the automatic recognition of veterinary diplomas across the EU, as well as the freedom for establishment of professionals and the freedom to provide a professional service wherever in the EU. These basic instruments for a functioning open market are now to be reinforced through the full application of the New Directive of mutual recognition of professional qualifications, where a benchmark of availability of high-quality professionals has been launched for the EU. Such a benchmark definition is of utmost importance for the EU as a whole, and particularly for the Establishments that impart the basic training for these professionals. However, if there is a dichotomy, as it is experienced at present, between the expected high-quality of the professionals and the qualitative aspects of the basic training, the system will no doubt crack in the long-term. It is a logical expectation that this is not the intention of the European Commission, and that provisions are to be taken to ensure this will never happen. However, a major responsibility lies on the establishments themselves and on the national authorities.

The Directive 78/1027 harmonises, as defined above, the minimum compulsory

subject and training requirements for the training of a veterinary surgeon within the EU but has, despite its 25 years of age, no description of neither qualitative nor quantitative aspects for these requirements, of utmost relevance for issues of quality assurance. There is, however, a very important statement in the EC-council Directive 78/1027/EEC (Annex of the Study programme for veterinary surgeons) where it is stated that "The distribution of the theoretical and practical training among the various groups of subjects shall be balanced and coordinated in such a way that the knowledge and experience listed in Article 1 (1) of this Directive *may be acquired in a manner which will adequately enable veterinary surgeons to perform all their various duties*".

Such a statement in the Directive lays the basis for quality assurance of veterinary training in the European Union at present. However, in order to warrant that the level of quality is the most appropriate, and to eventually achieve its highest possible and most relevant level among establishments in the EU, an analysis should be undertaken. Such analysis of the Directive has already been regarded as most relevant for the basic veterinary training and the development of the profession, by both the EAEVE and the FVE.

THE ROLE OF INTEREST ORGANISATIONS IN THE EVALUATION OF VETERINARY SCHOOLS

Owing to this apparent lack of clear internal demands for quality within the frame of the Directive 78/1027, and in order to be able to ensure that different establishments of veterinary education (among those EU-schools and those of candidate countries) are not only complying with the minimum requirements laid down by the Directive but also are aware of quality issues, a system of voluntary evaluation of teaching capabilities has been running since 1986. The system was firstly run by the EAEVE with participation from veterinary practitioners in the visitation teams and, since June 2000 by the joint efforts of the EAEVE and the FVE, following the adoption of a new set of Standard Operation Procedures (SOP, 2000). This SOP has been revised in 2001.

Basically all the EU-schools (exception made of those recently established) have been evaluated at least once. A total of 65 Faculties has to date been evaluated under the European system. This figure does not include the evaluations performed by the American Veterinary Medical Association (AVMA) or the Royal College of Veterinary Surgeons (RCVS). Evaluations are to be carried out at 7–10 year intervals for each school and some (6) are already to be scrutinised now for the second time. Most schools ($>90\%$) have expressed satisfaction with the present system of evaluation. The evaluation system has rendered rectification of major deficiencies (those defined as not conforming with the minimum requirements of the Directive 78/1027), and it has enabled schools to enact curricular changes derived from the recommendations, to acquire additional academic and support posts, refurbish buildings and

facilities, undertake new management structures and substantially augment their *numerus clausus* or otherwise, to ensure possible best-quality training. One wonders if these changes would have been possible without the aid of the voluntary evaluation system, whose recommendations actually help the responsible national authorities to understand the value of the veterinary training in a European perspective. The schools themselves should remember the evaluation has the ultimate aim to help the training establishment to improve, not to jeopardize, its existence.

Running this voluntary evaluation system, that has been perceived as very effective in providing useful assessment reports, has made possible the identification of not only deficiencies among schools but also shortages in the Directive 78/1027, particularly in issues of quality assurance. Therefore, for the sake of the European harmonisation everyone is willing to reach, a better and more transparent system is to be devised and applied. The public, including the pertinent national authorities and the potential students, need to know the contents of these assessment reports. The better knowledge of strengths and weaknesses are of importance to encourage staff and student mobility, also a major goal of the European Commission. Teacher mobility is far too low among establishments of veterinary medicine in Europe, and curricular development, to cite one reason, benefits from teachers' interaction. This mobility should be, and in some schools has already become policy, a pre-requisite to facilitate sustainable student mobility within the Erasmus programme.

AMENDMENTS TO THE DIRECTIVE/S: A NEED FOR FURTHER DEVELOPMENT

Various amendments to the Directive 78/1027 have been considered and proposed during the past 25 years, mostly concerned with the differences among the various establishments of veterinary education. These differences have been substantiated, as expected, following voluntary evaluation of teaching capabilities since 1986. The evaluation of the schools, standardised with the SOP, and the participation of both organisations EAEVE and FVE is a lengthy procedure that ends with the agreement between parties at the Joint Education Committee (JEC) and Executive Committees. Obvious differences exist among establishments, inherent of a European perspective, both in terms of facilities, input/output (students/graduates), numbers of teaching and support staff, accessibility to teaching material etc, all of which can cause eventual differences among the graduates.

Control mechanisms to ensure that the quality of the veterinary training is acceptable have been proposed by the existing Advisory Committee of Veterinary Training (ACVT, Internal Market III/F/5171/7/92-EN, Brussels 2–5–1993) and FVE (FVE/2000/011) but were overruled by the Commission for not fully considering the free-movement of professionals. The FVE, as a professional organisation, has earlier focused on issues of veterinary demography (Document FVE 00/011) as a major threat to the quality of veterinary education in Europe. However, such a focus on

issues of veterinary students or numbers of graduates among the EU has not impacted *per se* on the quality of training of the establishments, since the latter has not been taken direct account of, a matter for lengthy, not always constructive, discussions between EAEVE and FVE. The FVE, again acting as the professional organisation it is, focused on threats such as higher rates of veterinary under- or unemployment and the question of an over migration of veterinarians, as those causing damage to the profession. The first mentioned issue is a reality in some countries, where the number of establishments of veterinary training exceeds the expectations of the internal market. Their existence sometimes follows short-sighted political agendas (statistical treatment of unemployment, excessive offer of undergraduates without the demand of a market) or expression of regional autonomy, etc. The second issue is unsubstantiated for most of the EU, exception made of few countries, where large numbers of veterinarians cross the borders to provide services or establish themselves, following EU established laws (Rodriguez-Martinez, unpublished survey). Other matters, such as changes in gender balance, are a worldwide reality, and not necessarily related to the quality of undergraduate teaching.

Despite disagreement being present for some of the above aspects between the organisations, there is a large list of agreed points, where the FVE and the EAEVE have repeatedly declared their intention to join efforts for the best development of the quality of veterinary training in the EU. Among these points is the design of a curriculum that provides (a) basic veterinary knowledge (curriculum core), (b) differentiation in later areas (clinics, research, veterinary public health, etc) and (c) a suitable system for continuing professional development (CPD's) and specialisations within the umbrella of the European College system (see below).

QUALITY ASSURANCE OF BASIC VETERINARY TRAINING: HOW TO ACHIEVE IT?

The EAEVE has repeatedly declared its wish to update the educational standards of the EU-Directive 78/1027, following considerations – in agreement with the professional organisations FVE and RCVS – that they need to be expanded and upgraded to best reflect the benchmarks stated in the SOP. Discussions with representatives from the DG15 Internal Market earlier this year have resulted in the suggestion by the officials that EAEVE and FVE should need to make a formal submission to present possible amendments to the Directive/s. The Vet2020 Steering Committee and the Regional representatives of the project have recognised the inherent value of these signals and the need of an analysis to disclose the best way to attempt adaptations of curricula that follow the trends identified in the Vet2020 European survey. It was also the opinion of the Steering Committee and the Regional representatives of the VET2020 project (Meeting in Lisbon, March 1st 2003), that such an in depth analysis should be carried out to produce a document listing a series of proposals, embracing earlier initiatives from both EAEVE and FVE and related to the outcome

of the Vet2020 project, that would be considered as a starting point for expected, suitable upgrading of the EU-Directive 78/1027 and the New Directive on recognition of professional qualifications. Such upgrading (as amendments of the Directive 78/1027 and therein) must be considered as a way to consolidate the requirements of veterinary quality that best define the benchmarks for a well-functioning open internal market for veterinary services and the establishment of veterinarians anywhere in Europe.

For such a task, an ad-hoc group was created (R. Leiser, J. Leibetseder, W. Swannet, S. Cinotti, J. Soriano, D. Noakes & H. Rodriguez-Martinez, chairman) which was to co-opt a representative from EAEVE and another from FVE, to draft a proposal (to be ready by September 20th, 2003) intended *to stimulate the preparation, by EAEVE and FVE, of a joint formal document to be submitted to the European Commission aiming at the evolvement, with particular emphasis on quality assurance, of the educational standards set out by the Directive 78/1027 (Annex V).*

This document should necessarily incorporate the benchmarks provided by the EAEVE/FVE Standard Operations Procedure (SOP) for visits of the establishments of veterinary education in Europe. As pointed out already, this visitation system has provided an invaluable first step in the harmonisation of veterinary education in Europe. However, more instruments, issued by the Commission, are needed to further continue on this path of quality assurance among the establishments of veterinary education in the EU, including those present in the countries that are candidates for imminent or later inclusion in the Union. Some of these items have been included in the Vet2020 project, where an exploration of the market needs for different profiles of veterinarians the next 20 years, would help both the establishments of veterinary education and the veterinary profession in focusing on issues of training quality while developing harmonised veterinary curricula in Europe.

AMENDMENTS OF DIRECTIVE 78/1027 AND THE NEW DIRECTIVE FOR MUTUAL RECOGNITION OF PROFESSIONAL QUALIFICATIONS: IS A SET OF ACTIONS POSSIBLE?

The primary goal for any amendments of the Directive/s is to ensure the best possible quality of the basic training, as well as an evaluation system that makes possible the surveillance (and thus quality control) of this training. Such a system is already present (Joint EAEVE/FVE SOP) but there is a need for a series of updates of the EU regulations so that the organs running this voluntary evaluation system (Joint Education Committee and Executive Committees) have a stronger ability to force schools to follow their recommendations as well as providing the EU member-states with the power to enforce rectification. In order to ensure transparency of the system, a system of publication of the final reports from each evaluation performed is desirable, eventually leading to the publication of lists of approved (or even not yet approved) establishments. The General Assembly of EAEVE, the highest body for

decisions within the organisation, has already taken a very important first step in this direction at its meeting in Toulouse, France (May 22nd, 2003) approving, unanimously, the following declaration: "*The present Directive 78/1027 is considered as a starting point for upgrading this Directive, including the SOP and accreditation of veterinary establishments*".

Such changes can be difficult to make under the current exercise of consolidating the new Directive of mutual recognition of professional qualifications. The Legal Affairs Committee of the European Parliament voted on June 17th 2003 to accept the principle of the Commission's proposed directive to integrate into a single text, establishing a general system thought to simplify and modernise all seven current sector directives governing the mutual recognition of professional qualifications, including veterinary surgeon, general nurse, dental practitioner, midwife, architect, pharmacist and doctor. Noteworthy, although both EAEVE and FVE, as well as the rapporteur (Mr. S. Zappalá, EPP-ED-I) marked their belief that the current system of sector directives and corresponding specific systems have been successful and efficient and should therefore be maintained intact, the Committee voted in favour of the New Directive. Under this factual scenario, there is a clear need to prepare for amendments, see that a qualified majority vote is present, being indispensable that a certain number of Member States agree on the amendments, under the period of discussion of the issue in substance, that ought to start by September 2003. The veterinary profession has a clear preventive role for human health going hand in hand with issues of animal health *per se*. Amendments to the new Directive might be difficult to achieve and Vet2020 is of the opinion that EAEVE and FVE should concentrate efforts on issues of quality assurance of training in the first place, focusing on convincing as many governments of the Member States to support such a change.

There is, therefore, an urgent need for agreement on a minimum set of items to be considered, and ultimately agreed, by the EAEVE and the FVE in terms of quality assurance for the evolvement of veterinary education. The primary item to be considered is the attainment of three levels of veterinary education, as already contemplated in the recommendations by ACVT on February 10th, 1993:

Initial training (5–6 years of veterinary training) divided into a basic (core) curriculum for all students, allowing in the final years, a period of in-depth studies. The latter can be done by in-depth studies associated with a final thesis work and/or by the creation of differentiation in the final (clinical) years or by any other devised programme, following school-, national- or even regional initiatives. These steps do not exclude each other, and allow for in-depth studies in ANY area of basic veterinary training, without detriment of a general license to practice veterinary medicine (Full diploma).

Complementary training (specialisation). This system, based on resident-positions (inward) or open training (outward) is linked to the European Colleges (ECVS) or National organisations, ruled by National legislation.

Continuing education (CPD's) within the frame of the Schools, and/or associated bodies (FVE, Colleges, other associations or even enterprises).

The here defined basic, initial training, is the primary target for a system of evaluation that must warrant a certain degree of quality assurance. Such basic training is to be followed by specialist studies (or postgraduate studies of academic nature) for three or four more years. The basic training period could be divided into two levels, in order to, eventually, best allocate the intentions of the Bologna Declaration, provided the extent of the Declaration would include Veterinary training. However, considering the presence of clear national or regional differences with regard to the need, or even the value of such division of levels, this issue should be handled cautiously. At present, there is no need to introduce a degree of Bachelor of Veterinary Medicine other than for academic purposes (as an intermediate degree WITHIN the veterinary basic training programme), since such graduates are not needed on the present labour market, a pre-requisite for the enforcement of the Bologna declaration.

Both EAEVE and FVE should jointly stand behind a series of initiatives, primarily as a follow-up of the recommendations on the modification of Directive 78/1027 proposed by the ACVT on February 10, 1993 (Internal Market III/F/5171/7/92-EN, Brussels 2–5–1993). In particular, areas of public health, herd health and surveillance and clinical subjects should be priority, following the identification made through the project VET2020. Adoption and control of quali- and quantitative minimal training has to be issued, with the full adoption of proper indicators (as stated in the SOP) of practical training with adequate teaching supervision. Focus should, however, be put on quality of teaching rather than solely on arithmetical ratios.

More in detail, EAEVE and FVE should work together to:

Fully implement the SOP (Joint EAEVE/FVE), using the modification of Directive 78/1027 proposed by the ACVT on February 10, 1993 (Internal Market III/F/5171/7/92-EN, Brussels 2–5–1993) as a basic, albeit modifiable model.

Evolve this SOP, by ensuring quality control of the teaching of *every* subject of the initial veterinary training with an extra-commitment on the areas identified as expanding areas in the veterinary market (VET2020).

Implement a transparent system of publication of the Evaluation report after scrutiny of the SOP-guided evaluation by the EAEVE/FVE Joint Education Committee (JEC) and the Executive committees and thus considered as Final Reports of each establishment.

Request from the Commission and pertinent bodies that a provision of power by the pertinent authorities is issued, to enforce that minimum resources (economic or otherwise) are ensured for the best-quality of training and that *rectification is possible*, when the quality of training is not satisfactory for a period passing two years from the SOP-guided inspection (application of derogation clause [Article 56]).

Reclaim the need to have a SOP-inspection elicited before a new establishment of

veterinary education is allowed to operate in the EU (pre-requisite of the SOP-guided visitation). As well, written consent is to be required for open publication of the final report by the JEC.

Negotiate the development of a system of official recognition of specialisation in veterinary medicine, in the new EU-Directive (SLIM Directive, 2001/19/EC) pending approval.

FINAL REMARKS

The above set of points has to be seen as positive for the profession as such, and by the establishments of veterinary education in particular. In the first case, it has to be viewed without detriment in the EU-New Directive considering the (i) provision of services or (ii) establishment in a host state. However, it is pertinent to consider that host states should be able to require completion of areas, when quality assurance – provided by a revisited SOP – is not proven. Obviously, this marks the needs for the schools to improve, or maintain, high levels of training quality. Without this pre-requisite, any system of full mobility will always be shadowed by disbelief and, ultimately, counteract the goal of full recognition of veterinary qualifications through the EU.

SUGGESTED LITERATURE

ACVT recommendations on the modification of Directive 78/1027, on 10 February, 1993 (Internal Market III/F/5171/7/92-EN, Brussels 2–5–1993): Concerning the coordination of provision laid by Law, Regulation or Administrative Action in respect of the activities of veterinary surgeons.

Allman, S. (2002) The evaluation of Veterinary Training in Europe. The relationship between the FVE and the EAEVE.

Council Directive 78/1026/EEC, 18 December 1978: Concerning mutual recognition of diplomas, certificates and other evidence of formal qualifications in veterinary medicine, including measures to facilitate the effective exercise of the right of establishment and freedom to provide services. EEC Official Journal, L 362, 23/12/1978/, p. 0001–0006, 1978.

Council directive 78/1027/EEC, 18 December 1978: Concerning the coordination of provision laid by Law, Regulation or Administrative Action in respect of the activities of veterinary surgeons. EEC Official Journal, L 362, 23/12/1978/, p. 0007–0009, 1978.

Council Directive 89/48/EEC, 21 December 1988: General system for the recognition of higher education diplomas awarded on completion of professional education and training of at least three year's duration.

EAEVE's International Education Symposium: The European system of evaluation of veterinary teaching establishments, 1986–1999. Allman DM, ed., Vienna, Austria, 49 pp, 2000.

Evaluation of Veterinary Training in Europe. Manual of Standard Operating Procedures, 2000, European Commission, Directorate General XV/Advisory Committee on Veterinary Training (ACVT)/European Association of Establishments for Veterinary Education (EAEVE), 54 pp, 2002.

Federation of Veterinarians of Europe (FVE): Quality of veterinary training (FVE/00/011, 2000).

Halliwell R. The problems facing veterinary educators. In Allman DM, ed: Proc 1998 EAEVE Veterinary Education Symposium (Curriculum Planning for the 21st Century), Lisbon, 1999: 3–7.

Nomisma Spp (2002) Exploration of market needs for different profiles of veterinarians for the year 2020. Survey for the VET2020 project. (VET2020: Prospects for veterinarian profiles: requirements by the profesion and the society).

Pritchard WR, ed. Future directions for veterinary medicine. Pew National Veterinary Education Program. Durham, Duke University, NC, USA, 1989.
Rijnberk A. (1999) Veterinary curriculum in the 21st century: Segmented admission, tracking and electives. In Allman DM, ed: Proc 1999 EAEVE Veterinary Education Symposium (Curriculum Planning for the 21st Century), Lisbon, 1999: 53–55.
Rodriguez-Martinez, H. (2000) Veterinary teaching of bovine reproduction. Proc. XXI World Buiatrics Congress, Punta del Este, Uruguay, 6 pp (720/8).
Rodriguez-Martinez, H. (2003) Reflections upon trends in education and research in small animal reproduction in Europe. J. Vet. Med. Educ. (In press).
SLIM Directive-2001/19/EC, amending the Council Directive 89/98/EEC and 92/51/EEC (2002/0061, COD). Proposal for a Directive of the European Parliament and of the Council on the recognition of professional qualifications.
VET2020 (2002) Current aspects of veterinary profession and education in Europe, 93 pp, Lisbon.
VET 2020 (2003) Seminarium on "Future directions for veterinary education", 5th General Meeting of VET2020, Toulouse, May 24, 2003.
Working Party on Undergraduate Education. Reflections on the future of undergraduate veterinary education in Europe. In Allman DM, ed: Proc 3rd General Assembly of EAEVE, Brescia, Italy, 9 pp, 1990.

Veterinary Research Communications, 28 (2004) 45–46

An Analysis of the Veterinary Profession in Italy – Characteristics and Prospects

P. Bossi
ANMVI Chairman, Cremona, Italy
Correspondence: ANMVI Chairman, Palazzo Trecchi, via Trecchi 20, 26100 Cremona, Italy. info@anmvi.itwww.anmvi.it-
E-mail: p.boss@infinito.it

ANMVI (*Associazione Nazionale Medici Veterinari Italiani*), the National Association of Italian Veterinarians, is a professional association which was established just four years ago, but which already includes 15 Italian cultural and scientific associations, is an active part of the UEVP, and represents the impressive total of 10,000 Italian practitioners.

We have carefully assessed the VET2020 European study, but we have not stopped there – ANMVI commissioned Nomisma to further elaborate the situation in Italy via an additional survey of data on veterinary practice in Italy in 2003, thus updating the data from the preliminary survey included in VET2020 by a further four years. This was done above all to determine the current situation of our profession in Italy in particular, and to identify possible future developments in the sector and therefore occupational developments for the coming years with reference to 2020.

For a number of years now, our association has continued to express great concern over the crises and difficulties that our veterinary world experiences, and has urged the competent authorities and different components of the sector to act urgently in order to avoid situations that will only lead to the qualitative and ethical degeneration of our profession. Unfortunately, the results of this survey – conducted in great detail and very comprehensively by Nomisma – confirm that our concerns have always been correct and based on real issues. We need only add some data to realise that the veterinary world is in an extremely dangerous high-risk situation that requires immediate intervention. It is unacceptable that in the EU one veterinarian out of six is Italian, that 26.6% of veterinary medicine students are Italian, and that almost 20% of veterinary medicine faculties are located in Italy, but that in spite of this we are not even in the top rankings in Europe for zootechnic resources, for either pets or farm animals. In addition, a comparison of the 1999 data with the 2003 data reveals a 13% increase in total members of the professional rolls in just four years!

Occupational growth forecasts for 2020 are low in percentage terms for our sector, whereas total members of the professional rolls are set to double by 2020 unless we take immediate action. In this extremely difficult situation, we consider it unthinkable that the Italian university system be augmented by another degree course in veterinary medicine, in a university such as Catanzaro which does not achieve the minimum

faculty requirements provided for by the Ministry in terms of its existing faculties, and which totally lacks all the equipment necessary for a veterinary medicine faculty. It is sad to say that this opening has been made possible through the collaboration of three other Italian veterinary medicine faculties, which will undoubtedly do no favours either for the students of this new phantom faculty, or for veterinary practice at the national level.

As a professional association, we have no choice but to expose the absurdity of this situation in Italian university education, however here it is our intention to address mainly those aspects concerned with what follows training – i.e. professional access.

The right to study is both fundamental and guaranteed by our constitution, but the right to study cannot necessarily tally with the right to carry out a profession. This is precisely the situation in Italy at present – the traineeship and state examinations have become a formality, such that 99% of candidates pass these examinations (FNOVI data) after a six month traineeship which can be done before graduation, and which in many cases is reduced to a mere list of attendance signatures with no effective carrying out of practical work.

A profession such as the veterinary profession, which requires unflagging dedication, constant effort in terms of ongoing training, considerable investment in facilities, apparatus and equipment, and continual regulatory compliance – and all this without the financial reward found in other liberal professions – requires training for professional activities which differs from the current training programme.

Students who access the profession need to be sufficiently motivated and trained, whereas at the moment the veterinary sector is currently choked with professionals who have not been adequately trained to face up to reality, and who in many cases are prepared to trade off their professionalism in order to pick up scraps of work, or who after months or even years of underemployment are destined to seek employment in another sector in which they can use their basic biology training.

We are therefore committed to having an effective practical traineeship included in the pending reform of the professional rolls, which is soon to be debated in parliament. This practical traineeship should be of adequate duration and performance, with some form of financial reward if possible, and at its conclusion there needs to be a real examination, in line with the other professions.

The purpose of all this is not so much corporative, to protect veterinarians who are already working, so much as to ensure that the students who graduate from our faculties in the future will have a real chance of finding work that is worthy of their efforts.

Veterinary Research Communications, 28 (2004) 47–49

Italian University System has to Match Educational Needs in Adherence to European Standards and Professional Scenario

G. Penocchio
Federazione Nazionale degli Ordini dei Veterinari Italiani (FNOVI), Roma, Italy
Correspondence: Federazione Nazionale degli Ordini dei Veterinari Italiani (FNOVI), via del Tritone, 125, Roma, Italy
E-mail:gapenoc@tin.it

FNOVI (Italian Federation of Veterinary Orders), after considering the results of the survey carried out by Nomisma, aimed at designing future scenarios for the veterinary sector in the year 2020, on behalf of the European Medical Veterinary Schools, cannot but confirm its envisaged perceptions. In fact, in 2020 the professional scenario will be characterised by an excess of professionals who will inevitably have to compete in a market characterised by limited demand (i.e. decreasing employment growth). This is in stark contrast to the professional position currently on offer.

Italian professionals will further suffer from the following disadvantages, as listed below:

- A high number of professionals (the number of vets is likely to increase by 100% by 2020);
- Underemployment and unemployment (a stable employment scenario in Italy is likely to be achieved in an averagely longer timeframe as compared with other European countries);
- Envisaged market barriers for new professional profiles;
- A slight contraction of a few professional sectors (Vet sector growth in Italy in 2020 is likely to be less than 3%) due to a decrease in zootechnical production, and a lower production of animal-based food;
- Professional education which hardly matches market needs, and is also characterised by an overall lack of knowledge and specialisation.

Such a context is worsened further by a local anomaly: the existence of 13 Vet faculties as recorded 1997/98, has been raised to 14 with the Udine Faculty (although this is not eligible to confer the formal university degree, but this is currently compensated by the Catanzaro Faculty) correspond to 20% of all European Faculties. Furthermore, these match the total number of faculties in Slovenia, Slovakia, Norway, Hungary, the Czech Republic, Sweden, the Netherlands, Ireland, Finland, Austria, Greece and half of France. The total number of Italian students constitutes the 26.6% of all European students. Currently, in fact, one out of six European Vets is Italian.

Considering the Catanzaro case, if we consider (as we do) that the free circulation of professionals is based on similar educational pathways, the 14th Vet Graduation

Course held at the Faculty of Human Medicine c/o *Magna Graecia* University in Catanzaro can neither be explained nor justified.

MIUR (Italian Ministry of University and Research) communications, which in 2001 aimed to reassure that it was impossible to approve the new course due to "an overall lack of necessary resources, as indicated by the Communitary Law for additional initiatives", were confirmed – in 2002 – by the European Vet Medicine Association Report, stating a "negative feedback to the Catanzaro initiative". This negative feedback was also supported by the Conference of the Italian Vet Medicine Faculty Deans in 2001, which declined the activation of new Class (47/S) of Graduation Courses. Considering all the negative indications mentioned above, we certainly did not expect the surprising advice given by the National University System Evaluation Committee.

I invite you all to read that document, as it represents what should never be done. A careful analysis of the text highlights: the lack of an innovative project, the unavailability of a project aimed at restructuring the "Condoleo Farm" gifted by the Province to the University, the lack of a zootechnic Farm, of a pound, of labs, of a teaching hospital, and of a slaughter-house.

However, all these negative circumstances were, amazingly, considered the basis for a favourable decision. In other words, the new Catanzaro Faculty was to be equipped with all the necessary staff by means of conventions with other Italian faculties. This communication was followed by the D.M. of the 3rd of September aimed at authorising the Catanzaro Graduation Course as a not well identified "matter of urgency". We are aware of the resignation of the President of the Dean Conference (many thanks to Prof. Girardi for his seriousness and coherence), and the protest of the professional world.

From our perspective there are at least three issues to reflect on:

- it is necessary to create an independent evaluation Authority as soon as possible;
- instead of improving the existing Faculties (only 2 out of 14 actually reach European standards) by turning some of them into specialty schools, personal and local interests have been favored;
- the Catanzaro experience may give rise to other similar initiatives to the detriment of quality (something the Catanzaro Graduation Course surely cannot guarantee). This will eventually contribute to the dispersion of Italian professionals to the rest of Europe.

As to the issue of professional access, also reinforced by the political intention to update the qualifying examination by the end of this year, the Federation of Orders has envisaged the setting up of an exam aimed at certifying, in addition to adequate knowledge, professional ability or at least the attitude to reach a professional level.

The Federation of Orders does not envisage nor encourage the use of internal barriers or obstacles aimed at postponing or forbidding the access of newly qualified graduates into the market, but simply aims at guaranteeing the natural objectives of

a qualifying examination. Such objectives involve a system to monitor professional ability based on the application of identical standards throughout the national territory, and managed equally by universities and professionals.

This exigency is perceived by FNOVI as a current necessity despite the statement in the MIUR outlines that the veterinary sector is not characterised by any system distortion, provided the coherence of the national data (2718 qualified out of 2760 candidates, equal to 98.5%, and five faculties reporting qualification of 100% students).

As for the junior graduation courses, the professional world wonders what a graduate in "Science of breeding, hygiene and well-being of dogs and cats" is meant to do. The answer is supposedly provided by the course advertisement brochure, which states that: "S/he will support the practitioner vets in her/his professional activity, either in-office, at vet hospitals, dog-pounds, and public and/or private cat recovery houses".

We cannot avoid noting that:

- there is no need for such a professional profile, nor will the market ever be able to integrate it;
- there is an attempt to create new professions with no market requirement for them;
- there is no need to create additional unnecessary ghostly professional profiles;
- it is necessary to think about new professional profiles from an ethical perspective, provided that addressing people's energies toward pure illusions with no serious occupational perspectives is an injustice.

In conclusion, first of all it is necessary to establish a university system which is able to match educational needs, and to guarantee its adherence to European standards aimed at offering a wider and more diversified range of options and competence as compared to those currently available. We strongly wish to create a stable agreement between the university and the professional world in Italy, with the aim of achieving long-term professional co-operation.

Veterinary Research Communications, 28 (2004) 51–56

Shellfish Farming: Technologies and Production

G. Prioli
M.A.R.E. Soc. Coop. a r.l.
Correspondence: M.A.R.E. Soc. Coop. a, r.l., Via E. Toti 2, 47841 Cattolica (RN), Italia
E-mail: gprioli@coopmare.com

Keywords: Manila clam, mussel, shellfish farming

Abbreviations: lm, linear meters

INTRODUCTION

In Italy, about 269 companies are more or less actively involved in shellfish farming, using different methods and often having plurispecific productions. These companies employ nearly 4,000 people (Prioli, 2001). This system is characterized by a complex structure where the legacy of old traditions coexists with modern intensive farming techniques. The transformation process leading to new farming methods, which enabled farmers to go beyond the local sphere of activity and handmade production, was triggered in the second half of the 1980s by the introduction of a new species and a new technique.

The introduction into lagoon areas of the mid-Adriatic Sea of Manila clam (*Tapes philippinarum*) resulted in an excellent adaptation of this new species to the local environment, to the extent that it spread spontaneously and allowed farmers to move from rearing practices to management of production areas in a more or less controlled way. This allowed farmers to benefit from a resource that completely changed the production structure of the areas involved in this process, with significant social repercussions.

The advent of new offshore mussel farming technologies allowed farmers to conquer new spaces, in addition to the traditional farms, which were mostly located in coastal and lagoon areas, such as the Veneto lagoon, the Gulf of Trieste, the Gulf of Taranto, etc. This led to the setting up of numerous offshore production facilities, no longer constrained by environmental and health problems. So, today shellfish farming has become the main aquaculture activity in Italy, although production is almost exclusively limited to mussels (*Mytilus galloprovincialis*), Manila clams (*Tapes philippinarum*), and only small quantities of Grooved carpet shells (*Tapes decussatus*) and oysters (*Crassostrea gigas* and *Ostrea edulis*) (Prioli, 2001).

The aim of this work is to analyse the most recent data, thereby contributing to a wider knowledge of the present status of Italian shellfish farming through a short overview of the two most representative sectors, namely mussel farming and clam farming.

MUSSEL FARMING

In the year 2000, there were 204 companies owning mussel farms (Table I), mostly concentrated in some main production poles whose location depends on the hydrologic and trophic features of the settlements.

Some of the main production areas have a long-standing tradition; others have become more important after the introduction of offshore farms and the "conquest" of new areas at sea. The most important production areas of longer tradition include the Gulf of Taranto (Puglia), La Spezia (Liguria), the Veneto lagoon, the Phlegraean coast (Campania), and, in more recent times, the Trieste coastline (Friuli-Venezia Giulia), the Gulf of Olbia (Sardinia), the Emilia-Romagna region and along the Adriatic coast of Puglia. There is currently a positive trend towards the settlement of new production areas along the coast of those Italian regions offering more favourable conditions for mussel growing. At the moment, only two coastal regions are still totally lacking in mussel farms, namely Calabria and Basilicata.

In Italy, the mussel farming sector employs about 1,800 people, around 1,500 as fixed workers and around 300 as temporary workers, hired occasionally or on a seasonal basis. The average number of workers per company is therefore nine (Table I).

The majority of workers are obviously concentrated in the main production areas previously mentioned. The regions of Veneto, Emilia-Romagna, Sardinia and Puglia are considerably above the national average, which is mainly due to the presence of a large number of cooperative businesses.

TABLE I
Regional distribution of number of companies and workers in the mussel farming sector. The column on the right shows the average number of workers per company

Region	Companies	Total workers	Fixed workers	Seasonal workers	Workers/ Company
Abruzzi	1	7	5	2	7
Campania	12	62	62	0	5
Emilia-Romagna	19	367	268	99	19
Friuli-Venezia Giulia	24	50	50	0	2
Lazio	4	26	22	4	7
Liguria	68	107	107	0	2
Marche	6	32	13	19	5
Molise	2	8	8	0	4
Puglia	31	375	348	27	12
Sardinia	16	270	125	145	17
Sicily	1	2	0	2	2
Veneto	20	558	558	0	28
Total	204	1864	1566	298	Average = 9

Apart from some relatively small farms based on extensive use of artificial reef units and management of shoals of mussels on the seabed, Italian mussel farming essentially relies on three rearing systems, namely the fixed system, the single ventia long-line and "Trieste" long-line or multi ventia.

The fixed system is employed in lagoon areas or in sheltered coastal areas, and can be traced back to more ancient settlements. However, facilities have been through a gradual process of modernization, which in some cases coincided with the adoption of other systems. The regions with the highest number of settlements of this kind are Puglia, Emilia-Romagna and Liguria, although in Emilia these facilities, situated inside the Sacca di Goro Lagoon, are gradually being abandoned and progressively replaced by offshore surface long-lines.

Single ventia plants are relatively new, since most of them have come into use over the last 15 years, but they have quickly become the strong point of Italian mussel farming, accounting for nearly 75% of the lm presently available for cultivation. Indeed, this parameter has been identified as an indicator of production capacity, since the number of strings of mussels cultivated is basically related to the linear meters of long-lines available. As previously mentioned, these kinds of facilities are located at sea, since they offer excellent resistance against marine weather events, even those of significant magnitude.

The multi ventia plants are very common in Friuli-Venezia Giulia, where nearly all rearing systems are of this type, and in Puglia, Liguria and Sardinia, as well. Multi ventia long-lines originated along the Trieste coastline, where they experienced their greatest development in the 1980s, and are used in partially or totally sheltered areas.

In Italy there are about 2,000,000 lm of long-line available, with each company managing about 10,000 lm on average. The regions with the largest number of linear meters are Emilia-Romagna, Puglia, the Veneto, Friuli-Venezia Giulia and Sardinia, where the largest companies are also based (Table II).

In absence of any other official and reliable sources, the data regarding production in 2000 and presented herein were derived from a set of evaluations mainly based on the following: values reported by producers; values obtained on the basis of production capacity of farms; values obtained on the basis of the characteristics of the production area and the staff employed; estimates by local experts derived from trade flows; assessment of produce transferred to other farms. The result is about 85,000 tons of production, well below the value emerging from official statistics, namely 130,000 tons (Prioli, 2001). Apart from the figure itself, the data highlight the presence of production poles, although in some cases the values are overestimated because of some adult mussels that are put on the market after a short period of immersion in these farms. Puglia, the Veneto and Emilia-Romagna account for 80% of national production, followed by Friuli-Venezia Giulia and Sardinia. In this case, the figures do not include the production of spat, which is not always adequately reported by farmers and comes from three main areas, namely Puglia, the Veneto and Emilia-Romagna.

The mussels produced in the various farms are not placed uniformly on the market,

TABLE II
Regional distribution of linear meters (lm) used for mussel farming. For each Region, the minimum and maximum company dimensions in lm are indicated

Region	Total lm	Average lm per company	Minimum	Maximum
Abruzzi	18,000	18,000	18,000	18,000
Campania	41,288	3,441	300	10,000
Emilia-Romagna	631,150	33,218	6,000	200,050
Friuli-Venezia Giulia	186,440	7,768	400	35,800
Lazio	21,295	5,324	1,500	6,000
Liguria	49,042	721	275	12,648
Marche	55,500	9,250	2,500	25,000
Molise	46,000	23,000	22,000	24,000
Puglia	550,270	17,751	700	210,000
Sardinia	143,660	8,979	1,050	36,200
Sicily	600	600	600	600
Veneto	303,240	15,162	110	82,500
Total	2,046,485	10,032	275	210,000

since there are seasonal peaks, which can be more or less dramatic. At a national level, the large majority of farms market their produce between May and September, while between November and February the number of farms selling mussels is lower (Prioli, 2001).

More than 70% of the national production is sold by producers to wholesalers, while about 11% is sold directly to consumers or retailers. The amount intended for the processing industry is very low, being lower than 1%. Around 14% of the national production is destined for other farms, although the amount that is sent to other farms with the intermediation of wholesalers, which cannot be quantified, should also be added to this percentage (Prioli, 2001).

CLAM FARMING

Clam farming companies are concentrated in the principal Italian lagoon areas. It has been estimated that there are 54 of them, generally located in the Po plain and the Veneto area (Emilia-Romagna and Veneto regions) and in Sardinia (Table III). In the lagoons of the High Adriatic, clam farming is mainly based on the production of Manila clam (*Tapes philippinarum*), and some modest quantities of Grooved carpet shell (*Tapes decussatus*) coming from the "pialasse" of the Ravenna area (brackish lagoons connected to the sea). In Sardinian ponds the autochthonous species of *Tapes decussatus* is extensively harvested.

TABLE III
Regional distribution of number of companies and workers in the clam farming sector. The first column on the left shows the average number of workers per company

Region	Companies	Total workers	Fixed workers	Seasonal workers	Workers/ Company
Emilia-Romagna	14	1053	830	223	59
Friuli-Venezia Giulia	4	11	11	0	3
Sardegna	8	265	245	20	33
Sicilia	1	14	10	4	14
Veneto	27	1677	1673	4	58
Total	54	3020	2769	251	50

The clam farming sector employs quite a high number of people, for a total of 3020 workers, also including temporary and seasonal staff, mostly employed in the regions with the highest level of production, namely the Veneto, with 1677 workers and Emilia-Romagna, with 1053 (Table III).

Clams are cultivated on the seabed, over a total surface area of about 112,000,000 m^2, 50% of which is in the Veneto, 36% in Sardinia and about 9% in Emilia-Romagna (Prioli, 2001). It should be pointed out, however, that the surface area reported here is the one that can be directly ascribed to fish farming companies, which do have some kind of resource management practice. So, if we are also to consider public areas, which are not directly linked to any company, but where clams are collected anyway, often by unauthorized fishermen, then the figures will increase considerably, up to about 220,000,000 m^2 (Rossi *et al*., 2000).

Clams are currently reared using naturally-produced juveniles, and only in some special cases by purchasing spat from artificial breeding. Since the Manila clam has adapted so well to the Adriatic lagoon environments, this species is now very widespread in those areas and farming activities basically consist of managing shoals and leased areas. This task includes soil cleaning and preparation, transfer of spat, thinning, assessment of present biomass, fishery rotation and surveillance.

At present, there is only one commercial hatchery used for Manila clam seed production, situated in the Grado lagoon, which is connected with other areas employed for the successive growing stages.

A recent study (Rossi *et al*., 2000) has shown that the national production of *T. philippinarum* in 1999 was about 61,800 tons, also including the product harvested outside farming areas; 50,700 tons come from the Veneto areas, 9,300 from Emilia-Romagna and 1,800 from the lagoons in Friuli-Venezia Giulia.

In 1999, production of *T. decussatus*, almost exclusively from Sardinian ponds, was as high as 135 tons.

Grooved carpet shell (*T. decussatus*) and Manila clam (*T. philippinarum*) are marketed in different ways, due to their respective production and market values. The

former is generally destined for the local market, mostly for various forms of direct sale, with a more limited involvement of different players. The latter has a larger market and a broader trading process, whose main protagonists are wholesalers and retailing through big department stores.

REFERENCES

Prioli, G., 2001. Censimento nazionale sulla molluschicoltura. Unimar Osservatorio tecnico-biologico. (Unimar, Italy)

Rossi, R., Paesanti, F., Turolla, E., Pellizzato, M., Caramori, G., Rossetti, E., Zentilin, A., Melaku Canu, D., Solidoro, C., Pastres, R., De Leo, G., Donati, F., Zoppelletto, M. and Ceschia, G., 2000. Elementi di valutazione ecologica, economica e sociale per fronteggiare la flessione produttiva di vongole filippine nell'Alto Adriatico. (Technical Report prepared for the Ministero delle Politiche Agricole e Forestali, Italy)

Veterinary Research Communications, 28 (2004) 57–62

Defense Mechanisms in Farmed Marine Molluscs

P.G. Tiscar* and F. Mosca
Department of Comparative Biomedical Sciences, Faculty of Veterinary Medicine, University of Teramo, P.zza A. Moro 45, 64100 Teramo Italy
**Correspondence: Dipartimento di Scienze Biomediche Comparate, Facoltà di Medicina Veterinaria, P.zza A. Moro 45, 64100 Teramo Italia*
E-mail: tiscar@imi.vet.unite.it

Keywords: defense mechanisms, endocannabinoids, Heat Shock Proteins, hemocytes, molluscs, opioids, reactive oxygen species

Abbreviations: ACTH, adenocorticotrophic hormone; HSPs, heat shock proteins; IL, interleukin; LPS, lipopolysaccharide; LuCL, luminol-dependent chemiluminescence; POMC, pro-opiomelanocortin; proDyn, prodynorphin; proEnk, proenkephalin; ROS, reactive oxygen species; TNF, tumor necrosis factor

INTRODUCTION

In recent years, molluscs and their defensive mechanisms have been investigated with great interest, both for the ecological, social, economic, nutritional role of these organisms and for their many pathological microrganisms (Arzul *et al*., 2001). In addition, bivalves represent very important carriers of some human pathogens such as hepatitis A virus or *Vibrio* species. The effectiveness of the mollusc defense system can modulate the properties of infective microorganisms, so mollusc-microorganism interactions assume importance as human health risk factors (Genthner *et al*., 1999; Lacoste *et al*., 2001). Mollusc defense systems and their state of integrity also represent a suitable biomarker in the monitoring of polluted sites; for example, several studies indicated the possibility of a correlation between high metal levels and low rates of phagocytosis and, possibly, between release of high levels of oxygen radicals and low background of organic contaminants (Pipe *et al*., 1995). Finally, characteristics of the immune system of molluscs could represent a simplified model for understanding more complex and phylogenetically more advanced systems.

HUMORAL DEFENSE SYSTEM

Invertebrates possess aspecific and innate immune mechanisms, and they are lacking in immune memory following the first encounter with a pathogen. Molluscs have humoral and cellular immunity, and the humoral system is constituted by lysosomal enzymes, agglutinins, lectins and antimicrobial peptides. Nevertheless, cellular immunity seems to perform the main role in shellfish immune processes (Roch, 1999).

Lysosomal enzymes (β-glucuronidase, acid and alkaline phosphatase, lipase,

aminopeptidase and lysozime) are comprised especially in granular hemocyte lysosomes and their release into serum depends on cell degranulation during phagocytosis (Pipe, 1990). Lysosomal enzymes also possess digestive function as indicated by their abundance in the stylus and digestive gland. In fact, digestive and defense functions could act contemporaneously because filtered bacteria represent nourishment for marine bivalves, so enzymes present in the digestive tract hydrolyze microorganisms performing both functions at the same time. Agglutinins have been found in many mollusc tissues and these glycoproteins act as opsonins against different types of erythrocytes (hemagglutinins) and other cells such as bacteria, protozoa and algae (Chu, 1988). Lectins represent agglutinin-like molecules and are present in the hemolymph and in the hemocyte membrane; they have a specific opsonizing function in mollusc defense mechanisms such as hemocyte aggregation and foreign cell agglutination. In particular, lectins are specific for hemocyte and bacteria glycoconjugates, so promoting recognition of foreign cells. However, the biological significance of the different carbohydrate specificities is not yet known, in fact most invertebrate lectins are heterogeneous and may also bind other ligands (Tunkijjanukij *et al*., 1998). Antimicrobial peptides have been discovered in hemolymph and mussel hemocytes, and have been classified as defensins, mytilins, myticins and mytimycin. These compounds act on a rather broad spectrum of microbial organisms and their activity has been shown outside hemocyte or in phagocytosed bacteria (Mitta *et al*., 2000).

CELL DEFENSE SYSTEM

Hemocyte phagocytosis represents the main process of the entire cell defense system and is constituted by different phases including recognition, adhesion, ingestion, destruction and elimination of foreign cells. Mollusc hemocytes also have important functions in transport of nutrients, wound repair, and removal of catabolic products or pollutants (Feng, 1988). Hemocytes can be generally subdivided into two main types: hyalinocytes, small cells with poor cytoplasm containing few or no granules; and granulocytes, large cells with a very abundant granular cytoplasm. Granulocytes are those most responsible for phagocytosis and they can be classified into acidophilic, basophilic and neutrophilic cells. The presence of these two hemocyte types and, possibly, of their subpopulation has been demonstrated through microscopic, immunological and enzymatic analysis in several molluscs such as *Mytilus edulis*, *M. galloprovincialis*, *Mercenaria mercenaria*, *Ruditapes decussatus*, *Ostrea edulis*, *Crassostrea virginica*, *Cr. gigas* (Hine, 1999).

Different hemocyte types isolated from many molluscs have the common characteristic of phagocytosing foreign cells or particles using lysosomal enzymes and respiratory burst. Mollusc hemocytes respond to appropriate stimuli with a respiratory burst resembling that of mammalian phagocytes. The respiratory burst activity consists of ROS synthesis. Superoxide, the hydroxyl radical, singlet oxygen, hydrogen peroxide, hypohalides, halidamines, nitric oxide and the peroxynitrite anion represent

the most important ROS and all are very strong oxidants. ROS production during phagocytosis of immune stimulant yeast (*Saccharomyces cerevisiae*), laminarin, phorbol miristate acetate and microbial LPS has been demonstrated for many different mollusc hemocytes such as *Patinopecten yessoensis*, *Pecten maximus*, *Cr. virginica*, *Cr. gigas*, *O. edulis*, *Mya arenaria*, *M. mercenaria*, *M. edulis* (Roch, 1999), *M. galloprovincialis* (Arumugam *et al*., 2000), and *R. decussatus* (Tafalla *et al*., 2003). However, the exact role of ROS production remains to be clarified in natural and experimental infections with important mollusc pathogens (Roch, 1999). In fact, *O. edulis* and *Cr. gigas* hemocyte do not show LuCL during *Bonamia ostrae* ingestion. In vitro studies have shown that *Cr. virginica* hemocytes fail to produce LuCL during *Perkinsus marinus* phagocytosis, but the addition of osmotically killed and structurally intact *Perkinsus* sp. cells triggered a large LuCL response, comparable to that shown with other foreign particles such as zymosan (Anderson, 2001). *M. galloprovincialis* hemocytes do not produce LuCL during stimulation with *Vibrio tapetis* and Perkinsus-like organisms (*Pseudoperkinsus tapetis*) (Ordas *et al*., 2000).

The inability of hemocytes to inactivate pathogen organisms has negative effects on the health condition of molluscs and on the economic or commercial activity connected with marine farmed molluscs. Lysosome-phagosome fusion was not observed in hemocytes infected with *Bonamia* sp. and the lack of fusion may be due to membraneous vesicles originating from parasite lipoid bodies (Hine *et al*., 1994). *Bonamia* sp. is mainly phagocytosed by agranular hemocytes and the higher resistance shown by some oyster strains to the disease is attributed to a reduced number of these target cells, thus suggesting the possibility of producing less susceptible oyster broodstock (Naciri-Graven *et al*., 1998). The parasite *P. marinus* is responsible for widespread mortalities in oysters *Cr. virginica* farmed along most of the U.S. Atlantic coast. Oyster mortality usually peaks in late summer-autumn when combined high temperature ($>25^{\circ}$C) and high salinity (at least 12%) favor development of heavy infections (Oliver *et al*., 1998). High environmental temperatures affect and modify cellular and humoral activities and, at the same time, increase the metabolic activity of the parasite; so, the rate of killing may not be sufficient to cope with *Perkinsus* sp. multiplication (Chu *et al*., 1993). Some authors report that *P. marinus* actively produces factors that act as free radical scavengers or as inhibitors of ROS generation by *Cr. virginica* hemocytes (Anderson, 2001). In contrast *Cr. gigas* is not susceptible to *P. marinus* disease and it is hypothesized that the capacity of *Cr. gigas* to increase its protease inhibitors represents the key event in its resistance to parasite infection by neutralization of proteases secreted by *P. marinus*, thus preserving the oyster hemagglutinins from degradation. Such hemagglutinins will be ready to act as opsonins stimulating phagocytosis of parasites (Romestand *et al*., 2002).

ROS produced by the host during inflammation are highly oxidising compounds against host and microbial macromolecules. In particular, the hydroxyl radical, hydrogen peroxide and peroxynitrite anion have the most important role in protein denaturation. Damages on polypeptide chains evoke the synthesis of HSPs, both in host and in pathogen to repair denaturated proteins and to protect their respective

proteic pool from possible denaturation. A significant 70 kDa HSP synthesis in *M. galloprovincialis* hemocytes during *Vibrio alginolyticus* phagocytosis has been demonstrated, while *Escherichia coli* determines only a small increase in HSP 70. These results are explained by the different phagocytosis rates derived from the different mollusc behaviour on interaction with bacteria coming from dissimilar environments and confirm the existing relationship between phagocytosis and HSPs production (Tiscar *et al*., 1998). Furthermore, the diversity in HSPs expression at conditions of high environmental temperatures seems to be an important factor promoting pathogenicity in the *P. marinus–Cr. virginica* relationship (Tirard *et al*., 1995). Moreover, among the possible pollution biomarkers, the HSPs expression in molluscs has been proposed because the synthesis of these proteins is likely to be induced by a large number of chemicals (Bierkens, 2000).

CHEMICAL MEDIATORS

A new interesting approach in invertebrate immunity studies in recent years involves research into the molecules that act as chemical mediators between immune system cells. These compounds also seem to possess an important role in bilateral information exchanges between the immune and neuroendocrine systems.

These relationships are mediated by many types of molecules such as corticotropin-releasing hormone, ACTH, monoamines (epinephrine, norepinephrine and dopamine), glucocorticoids, free radicals, cytokines (IL-1, IL-6 and TNF-α), opioid peptides and opiates. Opioids are produced through proteolysis of large precursor molecules such as POMC, proDyn and proEnk. Effects of POMC derived peptides have been particularly noted in the vertebrate immune system, concerning antibodies synthesis, chemotaxis and phagocytosis (Stefano and Salzet, 1999). *M. edulis* hemocytes contain mammalian-like POMC and its derivates such as methionine-enkephalin-like peptides, γ-melanocyte-stimulating hormone, α-melanocyte-stimulating hormone, ACTH, β-endorphin and γ-lipotropin hormone (Stefano *et al*., 1999; Ottaviani *et al*., 1997). ACTH has been revealed in *M. edulis* hemolymph stimulated with LPS and selected ACTH fragments seem to modulate the mobility of hemocytes and, in parallel, to contrast stimulation of hemocytes with TNF-α. ProDyn and proEnk-like proteins have been characterized in *M. edulis* and proEnk, in particular, contain antimicrobial peptides such as enkelityn or peptides similar to peptide B and bovine amidorphin. The endocannabinoids receptor system has also been characterized in the mussel, together with opioids system. In fact, mussel hemocytes seem to possess a cannabinoid 1 receptor-like related to nitric oxide release. Briefly, peptide opioids give an amoeboid morphology to hemocytes, while the endocannabinoid anandamide inhibits this phenomenon through a nitric oxide dependent mechanism (Salzet and Stefano, 2002). The cytokines represent the most important immune mediators, playing an essential role in mechanisms such as cell multiplication and differentiation, apoptosis and cytotoxicity. IL-1α, IL-1β, IL-6, IL-8 and TNF-α have

been isolated in *M. edulis*. IL-1 and TNF-α influence the mobility of *M. edulis* hemocytes and an increase in IL-1-like molecules has been shown during contact of hemocytes with a synthetic opioid analogous to methionine enkephalin (Stefano *et al*., 1999; Ottaviani *et al*., 1997).

CONCLUSIONS

In conclusion, comparative studies on immune invertebrates systems could facilitate understanding of the receptors involved in innate immunity. These receptors evolved in higher organisms with the main function of influencing the "pain" signal originating from danger. In addition, understanding the mechanisms of the mollusc immune system represents an important research objective in the protection of shellfish production from pathogens and help us to better understand the main innate biological processes that have maintained their characteristics through the evolution of different organisms.

ACKNOWLEDGEMENTS

This work was supported by grants provided by the Italian Ministry for Agricultural and Forest Politics (VI Piano Triennale della Pesca e dell'Acquacoltura).

REFERENCES

Anderson, R.S., 2001. Reactive Oxygen Species and antimicrobial defenses of invertebrates: a bivalve model. In: Beck *et al.* (eds), *Phylogenetic Perspectives on the Vertebrate Immune System*, Kluwer Academic/Plenum Publishers, 131–139

Arumugam, M., Romestand, B., Torreilles, J. and Roch, P., 2000. *In vitro* production of superoxide and nitric oxide (as nitrite and nitrate) in *M. galloprovincialis* hemocytes upon incubation with PMA or laminarin or during yeast phagocytosis. *European Journal of Cell Biology*, **79**, 513–519

Arzul, I., Renault, T., Lipart, C. and Davison A., 2001. Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. *Journal of General Virology*, **82**, 865–870

Bierkens, J.G.E.A., 2000. Applications and pitfalls of stress proteins in biomonitoring. *Toxicology*, **153**, 61–72

Chu, F.-L.E. and La Peyre, J.F., 1993. *Perkinsus marinus* susceptibility and defense-related activities in eastern oysters *C. virginica*: temperature effects. *Diseases of Aquatic Organisms*, **16**, 223–234

Chu, F.-L.E., 1988. Humoral defense factors in marine bivalves. In W.S. Fisher (ed.), *Disease Processes in Marine Bivalve Molluscs*. American Fisheries Society Special publication 18, Bethesda, Maryland, 178–188

Feng, S.Y., 1988. Cellular defense mechanisms of oysters and mussels. In W.S. Fisher (ed.), *Disease Processes in Marine Bivalve Molluscs*. Fisheries Society Special publication 18, Bethesda, Maryland, 153–168

Genthner, F., Volety, A., Oliver, L. and Fischer, W., 1999. Factors influencing *in vitro* killing of bacteria by hemocytes of the eastern oyster (*Crassostrea virginica*). *Applied Environmental Microbiology*, **65**, 3015–3020

Hine, P.M., 1994. Interaction of phagocytosed *Bonamia sp.* with hemocytes of oysters *Tiostrea chilensis*. *Diseases of Aquatic Organisms*, **20**, 219–229

Hine, P.M., 1999. The inter-relationships of bivalve hemocytes. *Fish and Shellfish Immunology*, **9**, 367–385

Lacoste, A., Malham, S., Cueff, A. and Poulet S., 2001. Stress and stress-induced neuroendocrine changes increase the susceptibility of juvenile oysters (*Crassostrea gigas*) to *Vibrio splendidus*. *Applied Environmental Microbiology*, **67**, 2304–2309

Mitta, G., Vandenbulke, F., Noel, T., Romestand, B., Beauvillan, J., Salzet, M. and Roch, P., 2000. Differential distribution and defence involvement of antimicrobial peptides in mussel. *Journal of Cell Science*, **113**, 2759–2769

Naciri-Graven, Y., Martin, A., Baudc, J., Renault, T. and Gerard, A., 1998. Selecting the flat oyster *Ostrea edulis* for survival when infected with the parasite *Bonamia ostrae*. *Journal of Experimental Marine Biology and Ecology*, **224**, 91–107

Oliver, L.M., Fisher, W.S., Ford, S.E., Ragone Calvo, L.M., Burreson, E.M., Sutton, E.B. and Gandy, J., 2002. *Perkinsus marinus* tissue distribution and seasonal variation in *C. virginica* from Florida, Virginia and New York. *Diseases of Aquatic Organisms*, **34**, 51–61

Ordas, M.C., Novoa, B. and Figueras, A., 2000. Modulation of the chemiluminescence response of mussel (*M. galloprovincialis*) hemocytes. *Fish and Shellfish Immunology*, **10**, 611–622

Ottaviani, E., Franchini, A. and Franceschi, C., 1997. Pro-opiomelanocortin-derived peptides, cytokines and nitric oxide in immune responses and stress: an evolutionary approach. *International Review of Cytology*, **170**, 79–141

Pipe, R.K., 1990. Hydrolytic enzymes associated with the granular haemocytes of the marine mussel *Mytilus edulis*. *Histochemistry Journal*, **22**, 595–603

Pipe, R.K., Coles, J.A., Thomas, M.E., Fossato, V.U. and Pulsford, A.L., 1995. Evidence for environmental immunomodulation in mussels from the Venice lagoon. *Aquatic Toxicology*, **32**, 59–73

Roch, P., 1999. Defense mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture*, **172**, 125–145

Romestand, B., Corbier, F. and Roch, P., 2002. Protease inhibitors and haemoagglutinins associated with resistance to the protozoan parasite, *Perkinsus marinus*, in the Pacific oyster, *Crassostrea gigas*. *Parasitology*, **125**, 323–329

Salzet, M. and Stefano, G., 2002. The endocannabinoid system in invertebrates. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, **66**, 353–361

Stefano, G. and Salzet, M., 1999. Invertebrate opioids precursor: evolutionary conservation and the significance of enzymatic processing. *International Review of Cytology*, **187**, 261–285

Stefano, G., Salzet-Raveillon, B. and Salzet, M., 1999. *Mytilus edulis* hemolymph contains pro-opiomelanocortin: LPS and morphine stimulate differenting processing. *Molecular Brain Research*, **63**, 340–350

Tafalla, C., Gomez-Leon, J., Novoa, B. and Figueras, A., 2003. Nitric oxide production by carpet shell clam (*R. decussatus*) hemocytes. *Developmental and Comparative Immunology*, **27**, 197–205

Tirard, C., Grossfeld, R., Volety, A. and Chu, F., 1995. Heat shock proteins of the oyster parasite *P. marinus*. *Diseases of Aquatic Organisms*, **22**, 147–151

Tiscar, P.G., Marsilio, F., Torelli, M.E. and Gatti, A., 1998 The 70 kDa heat shock proteins expression in mussel (*M. galloprovincialis*) hemocytes is not only related to heat treatments but also to the phagocytosis of bacteria. *Bollettino Società Italiana Patologia Ittica*, **24**, 29–38

Tunkijjanukij, S., Giaever, H., Chin, C.C. and Olafsen, J.A., 1998. Sialic acid in hemolymph and affinity purified lectins from two marine bivalves. *Comparative Biochemistry and Physiology – B Biochemistry and Molecular Biology*, **119**, 705–713

Veterinary Research Communications, 28 (2004) 63–67

National and EU Pathologies and Norms in Mollusc Culture

G. Ceschia
Istituto Zooprofilattico Sperimentale delle Venezie, Area Ittiopatologia, Laboratorio Patologia Molluschi, Via della Roggia, 102, 33030 Basaldella di Campoformido (UD), Italy
Correspondence: Istituto Zooprofilattico Sperimentale delle Venezie, Area Ittiopatologia, Laboratorio Patologia Molluschi, Via della Roggia, 102, 33030 Basaldella di Campoformido (UD), Italy
E-mail: ceschiag.izsv@libero.it

ABSTRACT

In the 1990s EEC countries drafted several directives concerning the aquaculture sector, including Directive 91/67/EEC of 28.01.1991 (accepted into Italy by DPR n. 555 on 10.12.1992) and Directive 95/70/EC from 22.12.1995 (accepted into Italy by DPR n. 395 on 20.10.1998). DPR 555 includes among its provisions a list of the diseases that are so serious for production that they must be eradicated from the EEC territory (Annex A, List I), as well as a list of other diseases that also affect production (Annex A, List II).

Annex A, List II of DPR 555, includes two mollusc diseases, namely Bonamiosis and Marteiliosis for flat oysters (*Ostrea edulis*). DPR 395 includes among its provisions that farm owners register their farm with the National Health Service and possess a loading and unloading register of the material introduced from or destined for another farm for reintroduction.

When the National Health Service finds a case of unusual death in the wild or in farms, it will apply a sanitary monitoring programme to ascertain or exclude the presence of the diseases included in Annex A to 91/67/EEC or Annex D to 95/70/EC as the cause of death. On February 5th 2003 the EU Commission modified Annex D (2003/83/EC).

Keywords: legislation, mollusc pathology, sanitary control

Abbreviations: DPR, Presidential decree

In the 1990s EEC countries drafted several directives concerning the aquaculture sector, including Directive 91/67/EEC from 28.01.1991, accepted into Italy by DPR n. 555 on 10.12.1992 ("concerning the animal conditions governing the placing on the market of aquaculture animals and products") and Directive 95/70/EC from 22.12.1995, accepted into Italy by DPR n. 395 on 20.10.1998 ("introducing minimum Community measures for the control of certain diseases affecting bivalve molluscs"). These norms have been and are still being continuously reviewed to meet the needs of this dynamically evolving sector.

DPR 555 includes among its provisions a list of the diseases that are so serious for production that they must be eradicated from the EEC territory (Annex A, List I), as well as a list of other diseases that also affect production (Annex A, List II).

The countries must become aware of the diseases that are present in their territories,

to implement sanitary measures to limit their spread as much as possible and to implement eradication plans (acknowledging the areas or farms that are free from one or more diseases).

To avoid the propagation of these diseases on EEC territory, the directive regulates how to reintroduce and market live animals according to the sanitary qualification of the area or farm. Special attention is given to aquaculture animals and products imported from third countries, for reintroduction, which must offer similar sanitary guarantees to those existing in EEC countries.

Annex A, List II of DPR 555 derived from Annex A of 91/67/EEC, includes two mollusc diseases, namely Bonamiosis and Marteiliosis for flat oyster (*Ostrea edulis*) (Table I).

Bonamiosis (also known as microcell disease and haemocyte disease of flat oysters), present in the Northern hemisphere, is caused by *Bonamia ostreae*. Besides *Ostrea edulis*, it can also infect other oyster species (*O. conchaphila*, *O. puelchana*, *O. angas*i and *Tiostrea chilensis)*.

Bonamiosis is present in Europe along the Atlantic coast from Spain to the United Kingdom and in the USA. Most infected oysters generally look normal. The pathological alterations are represented by haemocyte infiltrations in various organs, in the digestive gland, the gonads, the gills and the mantle. The parasite multiplies in the haemocytes that phagocytosed it; when the haemocytes are affected by necrosis, most parasites can be released.

The disease is transmitted from oyster to oyster. The initial phase can affect oysters of all ages. In the presence of an endemic disease, bigger oysters are most likely to be affected.

The disease is present all year round and the induced death rate can reach 90%, increasing in the summer period. Stress conditions on the molluscs (turbidity, manipulation, etc.), as well as high concentration of oysters affect the appearance of the disease and its consequent mortality rate. No eradication systems have yet been developed. The mortality rate may be reduced by using a floating farming system and/or decreasing the population density.

To market the product, in acknowledged areas and farms, EEC norms require a thorough sanitary control of the introduced material (to ensure it is free from disease). If *Crassostrea gigas* is introduced into acknowledged areas or farms, it must be carefully checked, especially if small, to avoid the presence of *O. edulis*.

In Italy, where there are currently no flat oyster farms (apart from a few mainly

TABLE I
91/67/EEC, Annex A: listed diseases/pathogens of molluscs

Disease	Pathogen	Susceptible host species
Bonamiosis	*Bonamia ostreae*	*Ostrea edulis*
Marteiliosis	*Marteilia refringens*	*Ostrea edulis*

experimental attempts), *Bonamia ostreae* is found sporadically and only in the wild. This situation is probably caused by farming attempts performed in the 1970s–1980s, that made use of infected foreign material, which infected the subjects present in the wild in the Adriatic Sea (Tiscar *et al*., 1991; Tiscar *et al*., 2003).

Flat oyster Marteiliosis is caused by *Marteilia refringens*, a Phylum Paramyxea protozoan. This disease is also known as Aber disease. It is present in Europe along the Atlantic coasts from Portugal to the United Kingdom. The infected oyster may look emaciated, will consequently slow down its growth and possibly die. The pathological changes include the presence of the parasite at various developmental stages, namely in the stomach epithelial cells, in the intestine, in the gills, but especially in the digestive gland tubules. The disease is transmitted naturally in oysters. The most favourable period for infection occurs in the summer, when the water temperature averages 17°C. Since no eradication system is devised, it is recommended not to transfer infected material to acknowledged areas or farms.

In Italy the situation is comparable to that previously described for Bonamiosis.

DPR 395 includes among its provisions the requirement that farm owners register their farm with the National Health Service and possess a loading and unloading register of the material introduced or destined for another farm for re-introduction. When the National Health Service finds a case of unusual death in the wild or in farms, it will envisage a sanitary monitoring programme to ascertain or exclude the presence of the diseases included in Annex A to 91/67/EEC or Annex D to 95/70/EC as the cause of death. On February 5th 2003 the EU Commission modified Annex D (2003/83/EC) (Table II).

Annex D originally included serious diseases not present in Europe. EU countries had to pay great attention not to introduce them, considering how difficult it is to carry out eradication plans in mussel culture. In the event of an introduction, it is compulsory to notify the EU of the centre of infection. Annex D was reviewed to adapt EU norms to OIE norms. In the annex to DPR 555 and DPR 395 all notifiable or meaningful diseases present in the OIE norms have now been included. As a consequence, many of these pathogens are present in Europe since some species are widely farmed or present in the wild (*Ostrea edulis*, *Crassostrea gigas*, *Ruditapes philippinarum* and *Ruditapes decussatus*). In this paper, only Perkinsiosis, present in Italy, will be briefly illustrated.

Perkinsiosis is caused by a protozoan of the *Perkinsus* genus that, according to recent studies, belongs more to the Dinoflagellata class than Apicomplexa. In Europe it was reported as *Perkinsus atlanticus* in several mollusc species, especially Veneridae (*Tapes decussatus*, *Tapes philippinarum*, *Venerupis pullastra*, *Venerupis aureus*). In Italy it was detected in farmed *Tapes philippinarum* (Ceschia *et al*., 1991) and in several wild mollusc species: *T. decussatus* and *V. aureus* (Da Ros *et al*., 1985, 1986; Breber, 1985); *O. edulis* (Tiscar *et al*., 1992), *Chamelea gallina* (Rubini *et al*., 1996) and *Callista chion*e (Canestri-Trotti *et al*., 2000). Recent genetic studies have shown that *P. atlanticus* is similar to *P. olseni* which infects several *Haliotis* species farmed in Australia, New Zealand, etc.

TABLE II. 2003/83/EC, Annex D: listed diseases/pathogens of molluscs

Disease	Pathogen	Susceptible host species
Bonamiosis	*Bonamia exitiosus* *Mikrocytos roughley*	*Tiostrea* chilens*is* *Ostrea angasi* *Saccostrea commercialis*
Marteiliosis	*Marteilis sydneyi*	*Saccostrea commercialis*
Microcytosis	*Mikrocytos mackini*	*Crassostrea gigas* *Crassostrea virginica* *Ostrea edulis* *Ostrea conchaphila*
Perkinsiosis	*Perkinsus marinus*	*Crassostrea virginica* *Crassostrea gigas*
	Perkinsus olseni/atlanticus	*Haliotis ruber* *Haliotis cyclobates* *Haliotis scalaris* *Haliotis laevigata* *Ruditapes philippinarum* *Ruditapes decussatus*
MSX Disease	*Haplosporidium nelsoni*	*Crassostrea virginica* *Crassostrea gigas*
SSO Disease	*Haplosporidium costale*	*Crassostrea virginica*
Withering syndrome of abalones	*Candidatus Xenohaliotis californiensis*	Members of the genus *Haliotis* including black abalone (*H. cracherodii*) *red abalone* (*H. rufescens*) *pink abalone* (*H. corrugata*) *green abalone* (*H. fulgens*) *white abalone* (*H. sorenseni*)

The mollusc can probably be infected by every developmental stage of the parasite, although the most effective stages are probably trophozoites and zoospora. The parasite reaches the host's connective tissue by lysis of the basal lamina of the external epithelia (gills, mantle, digestive gland, as well as kidney, foot, gonads, intestine) or may be spread through the hemolymph. Infected molluscs do not normally present any clear external clinical characteristics, but in the case of a serious infection where the subjects may be present pale pulp colouring and weak consistency, greyish gills due to the presence of whitish nodules (parasitical cysts) may be observed.

The presence of *Perkinsus* negatively affects numerous structures and physiological activities of the host causing alterations in the gonads, tissue degradation and disorganization, influencing tolerance to salinity, causing acidosis and limiting growth. At

present researchers pay particular importance to this parasitosis. A large infection, in Europe, may cause mortality by itself. Its presence, even if limited, when the molluscs are stressed, increases the death rate. Korean researchers believe that *P. atlanticus* has damaged and drastically reduced clam production in Korea. High temperatures and salinity increase the damage and the intensity of the infestation. The host responds with an increased inflammatory reaction represented by a granular-type hemocyte infiltration. The parasite may be isolated (free or intrahemocytal) or enclosed in cysts made of a positive PAS substance.

REFERENCES

Breber, P., 1985. On-growing of the carpet-shell clam (*Tapes decussatus*) (L.): two years experience in Venice Lagoon. *Aquaculture*, **44**, 51–56

Canestri-Trotti, G., Baccarani, E.M., Paesanti, F. and Turolla, E., 2000. Monitoring of infections by Protozoa of the genera *Nematopsis*, *Perkinsus* and *Porospora* in the smooth venus clam *Callista chione* from the North-Western Adriatica Sea (Italy). *Diseases of Aquatic Organisms*, **42**, 161–257

Ceschia, G., Zentilin, A. and Giorgetti, G., 1991. Presenza di *Perkinsus* in vongole veraci (*Ruditapes philippinarum*) allevate nel Nord-Est Italia. *Bollettino della Società Italiana di Patologia Ittica*, **5**, 101–108

Commission Decision 2003/83/EC of 5th February 2003 amending Council Directive 95/70/EC introducing minimum Community measures for the control of certain diseases affecting bivalve molluscs. OJ No. L 32, 07.02.2003

Council Directive 91/67/EEC of 28th January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products. OJ No. L 46. 19.02.91

Council Directive 95/70/EC of 22nd December 1995 introducing minimum Community measures for the control of certain diseases affecting bivalve molluscs. OJ No. L. 332, 30.12.1995

Da Ros, L. and Canzonier, W.J., 1985. *Perkinsus*, a protistan threat to bivalve culture in the Mediterranean basin. *Bulletin of European Association of Fish Pathology*, **5**(2), 23–27

Decreto del Presidente della Repubblica 20 ottobre 1998, n. 395: Regolamento recante norme di attuazione della direttiva 95/70/CE in materia di misure minime di lotta contro talune malattie dei molluschi bivalvi. *Gazzetta Ufficiale della Repubblica Italiana*, Serie generale n. 270 del 18 novembre 1998

Rubini, S., Cappellaro, H., Alessi, A., Badini L. Campanini, A. and Marzadori, F., 1996. *Perkinsus* sp. nei bivalvi dal Reno a Cesenatico. *Laguna*, **1**(96), 25–31

Tiscar, P.G., Zizzo, N. and Tempesta, M., 1991. Su alcune patologie riscontrate in ostriche piatte (*Ostrea edulis*) provenienti da banco naturale. *Bollettino della Società Italiana di Patologia Ittica*, **7**, 13–18

Tiscar, P.G., Chagot, D., Troncone, A., Desantis, S. and Marsilio, F., 1992. Pathologies found in molluscs from Apulian coasts, Southern Italy. *5th International Colloquium on Pathology in Marine Aquaculture*, 2–4 April 1992, Montpellier France, 77

Tiscar, P.G., Quaglio, F., Della Salda, L., Ceschia, G., Delgado, M.L. and Restani, R., 2003. Presenza di *Bonamia ostreae* in ostriche piatte (*Ostrea edulis*) del Nord Adriatico. *Bollettino della Società Italiana di Patologia Ittica*, **35**, 2–10

Veterinary Research Communications, 28 (2004) 69–74

Bivalve Molluscs: Productivity in the Campania Region and Related Sanitary Aspects

A. Anastasio[1]* and E. Fariello[2]
[1] *Department of Zootecnic Sciences and Food Inspection, Faculty of Veterinary Medicine, University of Naples "Federico II", via Delpino 1, 80136 Naples, Italy;*
[2] *ASL NA1 P. zza Municipio Naples, Italy*
* *Correspondence: Dipartimento di Scienze Zootecniche e Ispezione Alimenti – Facoltà di Medicina Veterinaria, Via Delpino 1, 80136 Napoli, Italia*

Keywords: bivalve molluscs, Campania region, productivity, viral contamination, new regulation

INTRODUCTION

The fishing activity in the Campania region has its roots in a very old tradition and is favoured by a large combination of factors: the sea, the coast and especially the geological phenomena of the Flegrei lakes.

In Roman times the Flegrei lakes belonged to the State. In that period the Lucrino and Averno lakes were fused together and because of their location they were chosen as the port of the imperial fleet. At that time the first fishing activities started and fishponds were built.

Fishing activities achieved high levels in productivity and wealth very quickly, but in 1538 everything suddenly stopped because of a volcanic eruption. Only in 1770 under Ferdinando IV of Borbone did the fishing activity receive a vital new impetus. Now these lakes are thoroughly exploited for aquaculture and oyster and mussel cultures.

In 1973 the mussel culture suffered an abrupt interruption because of a cholera epidemic. All fishponds of the Naples gulf were destroyed as the source of the epidemic was attributed to mussels. Subsequently this activity recovered slowly and today there is a large number of fishponds on the east coast of Naples (Torre del Greco, Torre Annunziata, Castellammare di Stabia), on the west coast (Baia, Cuma, Bacoli, Villa Literno) and on the Naples gulf. The most important fishponds are located:

(a) in Baia, with an area of around 16 square kilometers. The production declared for the last year was of about 9500 quintals mussels;
(b) in Castellammare di Stabia with an extension of 1.6 square kilometres and declared production of about 1000 quintals;
(c) in Torre del Greco where the fishponds cover an area of about 0.8 square kilometers with a declared production of 250 quintals;
(d) in Fusaro lake the surface dedicated to mussel culture is around 0.3 square kilometers and the official production is of about 2500 quintals.

The official average annual mussel productivity of the Campania region is of about 15,000 quintals which are mostly absorbed by the local market. However assessments by the ASSOMITILI (the most important producers association) show a real production of around 30,000 quintals.

These data highlight the notable difference between the potentiality of the fittings and the declared production. Therefore a notable quantity of product is introduced into the market illegally without sanitary controls. Neither the sanitary authorities nor the policy authorities are able to control such massive abuse.

In Campania there are nine bivalve mollusc dispatch centres and five dispatch/purification centres and the species of bivalve molluscs commonly commercially exploited include Mediterranean blue mussels (*Mytilus galloprovincialis*), various clams including the native clam (*Tapes decussatus*), manila clam (*Tapes philippinarum*), razor shell clam (*Ensis* spp.), flat oyster (*Ostrea edulis*), donax clam (*Donax trunculus*) and the striped venus (*Venus gallina*).

Bivalve molluscs can be the vehicles or agents of different parasitic, bacterial and viral diseases as well as biotoxins and contaminants (Jaksic *et al*., 2002; Chironna *et al*., 2002).

Although indigenous marine viruses are very abundant, only human viruses have been associated with illness due to seafood consumption. Viral problems are limited to the role of food in recycling human viruses back to humans. The viruses most adapted for this purpose are those transmitted by the fecal-oral route and include viral agents causing human gastrointestinal diseases such as hepatitis A virus (HAV) and the polio virus. These viruses contaminate shellfish through water contamination (i.e sewage pollution of the marine environment), or inadequate hygiene practices during processing and handling (Lees, 2000).

Virus accumulation by shellfish is a complex process and depends on several factors among which temperature is one of the most important. Viral and bacterial accumulation under natural conditions changes significantly through the year. In clams the greatest accumulation of microorganisms occurs at temperatures ranging from 11.5 to 21.5°C (Jofre, 1992).

The quality of shellfish depends on the quality of the growing waters and on the process of the shellfish self-cleaning mechanism (depuration). These factors are considered to be effective for the reduction or elimination of bacteria but they are not very successful in preventing viral transmission (Jofre, 1992).

Mussels are the most important disease vectors in Campania region. The association of infectious illnesses with this species reflects the traditional consumption of raw or lightly cooked mussels.

Shellfish bacterial contamination is currently evaluated by means of coliforms, *E. coli* and *Salmonella* spp. counts according to the EU shellfish microbiological standards. However, bivalve molluscs classified as moderately polluted according to EU standards have been found to be contaminated with HAV and enterovirus. In England an outbreak of viral gastroenteritis (Norwalk agent) was caused by the

ingestion of oysters that had previously been depurated according to EU sanitary regulations.

In the Naples area, since 2000 the Veterinarian Services have introduced strong controls against the illegal sale of molluscs as a consequence of an increase of HAV outbreaks observed by the Epidemiological and Prophylaxis Service. In that year 15 samples of bivalve molluscs were examined for the presence of HAV.

Ten samples were negative for HAV while the remaining 5 samples (3 mussels, 1 donax clam and 1 manila clam) were found to contain viral RNA coming from a whole virus. One of these samples came from the Campania region.

A similar sampling was carried out during 2001. Three samples (one mussel and two clams) out of nine were found to contain viral RNA coming from a whole virus. At the same time the Veterinarian Service carried out a microbiological screening on shellfish. One sample from the Lazio region out of a total of 29 was found not to conform to all EU microbiological standards. These results reinforce once again the inadequacy of bacteriological standards in the assessment of viral contamination.

In the revised draft Regulation of the European Parliament and of the Council laying down specific hygiene rules for food of animal origin the bivalve molluscs chapter is one of the most important.

This Regulation lays down general rules for food business operators on the hygiene of foodstuffs, taking into particular account the fact that the primary responsibility for food safety rests with the food business operator and that he needs to ensure food safety throughout the food chain, starting with primary production. The provisions of this Regulation apply to primary production and the associated operations (transport, storage and handling of primary products at the place of production, provided that this does not substantially alter their nature). As far as possible, food business operators, by the means of guides to good practice and the application of HACCP principles, have to ensure that primary products are protected against contamination (taking into consideration any processing that primary products will subsequently undergo).

In Chapter Two (Hygiene requirements for the production and harvesting of live bivalve molluscs) several requirements were established for (a) production areas, for (b) harvesting and handling after harvesting and (c) for relaying. In the case of production areas the requirements are similar to those fixed by Directive CE 492/91. In addition permitted treatment methods are specified, such as sterilisation in hermetically sealed containers and heat treatment, that live bivalve molluscs must undergo to eliminate pathogenic microorganisms, when they have not been submitted to purification or relaying. In addition food business operators must not produce live bivalve molluscs in, or harvest them from, areas that the competent authority has not classified, or which are unsuitable for health reasons. Food business operators must take into account any relevant information concerning areas suitable for production and harvesting, including information on environmental and weather conditions. They must use this information to determine the appropriate treatment to apply to harvested batches. For harvesting and handling after harvesting food business operators

have to conform to other requirements, such as adequate protection of live bivalve molluscs from crushing, abrasion or vibration and against extreme temperatures and preventing their re-immersion in water that could cause additional contamination. When carrying out conditioning in natural sites, they have to use only areas that the competent authority has classified as being of class A. There must be a minimum distance between relaying areas, and also between relaying areas and production areas, so as to minimise any risk of the spread of contamination. The food business operator must use techniques for handling live bivalve molluscs intended for relaying that permit the resumption of filter-feeding activity after immersion in natural waters. The relaying period must be fixed depending on the water temperature. This period must be of at least two months' duration unless the competent authority agrees to a shorter period on the basis of the food business operator's risk analysis. The 'all in, all out' system must be used, so that a new batch of molluscs cannot be brought in before the whole of the previous batch has been removed.

Finally, the food business operators managing relaying areas must keep permanent records of the source of live bivalve molluscs, relaying periods, relaying areas used and the subsequent destination of the batch after relaying, for inspection by the competent authority.

Another important chapter is the Fifth (health standards for live bivalve molluscs) in which it was fixed that, in addition to ensuring compliance with the microbiological criteria adopted in accordance with the Regulation on the hygiene of foodstuffs, the food business operators must ensure that live bivalve molluscs placed on the market for human consumption meet the known standards. Many of these standards are the same as those in the current legislation, such as an adequate response to percussion and normal amounts of intravalvar liquid. In addition molluscs must not contain marine biotoxins in total quantities (measured in the whole body or separately in any edible part) that exceed the following limits: for Paralytic Shellfish Poison (PSP), 800 micrograms per kilogram; for Amnesic Shellfish Poison (ASP), 20 milligrams of domoic acid per kilogram; for okadaic acid, dinophysistoxins and pectenotoxins together, 160 micrograms of okadaic acid equivalents per kilogram; for yessotoxins, 1 milligram of yessotoxin equivalent per kilogram and for azaspiracids, 160 micrograms of azaspiracid equivalents per kilogram (Proposal for a Regulation of the European Parliament and of the Council laying down specific hygiene rules for food of animal origin, 2002).

In our opinion, and in agreement with other authors (Jofre, 1992), to reduce public health hazards associated with the consumption of shellfish, two kinds of measures should be considered: those regarding consumer education and information and those aimed at reducing the contamination to the lowest possible levels.

It is important to educate the public (consumers, food manipulators) and high risk groups (i.e. diabetics, immunodeficients) about the risks deriving from consumption of raw or undercooked shellfish. This custom is one of the most frequent risk factors for acquiring HAV for all age groups in our country (Mele *et al*., 1997). But it is also important to implement the procedures based on the HACCP principles, which,

together with the application of good hygiene practice, should reinforce the responsibility of food business operators. Moreover, the present regulations must be enforced, for example through the introduction of new indicator organisms. New techniques, like molecular biology, for virological analysis of shellfish have to be adopted to establish the safety of growing water and the efficacy of the depuration process. These techniques must be adequately validated before routine use.

Finally interdisciplinary collaboration between different sectors of food hygiene is important to guarantee an effective control. With regard to this it is interesting to notice that in the year 2000 the researchers of the Zoological Station in Naples identified a saxitoxin and a neosaxitoxin in a microseawood (genus *Alexandrium*) (Fig. 1) as well as domoic acid in a diatom (genus *Pseudo-Nitzschia*) (Fig. 2) collected in the Naples Gulf. Since then the veterinarian Service has been checking for the presence of these toxins in samples of bivalve molluscs sold in town. At the moment all results are negative.

Figure 1. *Alexandrium minutum*

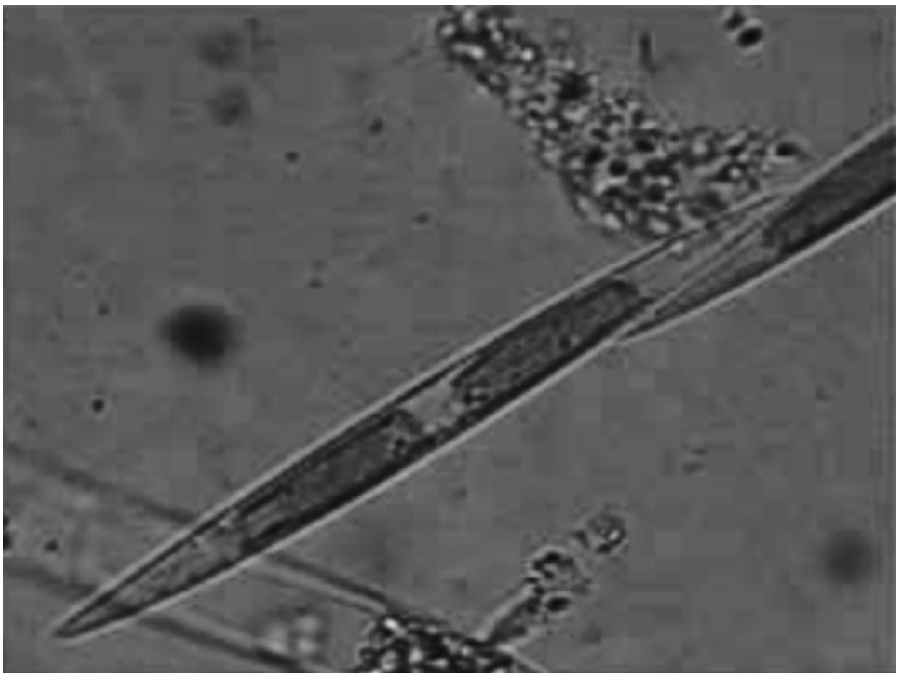


Figure 2. *Pseudo-Nitzschia seriata*

REFERENCES

Chironna, M., Germinario, C., De Medici, D., Fiore, A., Di Pasquale, S., Quarto, M. and Barbuti, S., 2002. Detection of hepatitis A virus in mussels from different sources marketed in Puglia region (South Italy). *International Journal of Food Microbiology*, **75**, 11–18

Lees, D., 2000. Viruses and bivalve shellfish. *International Journal of Food Microbiology*, **59**, 81–116

Jaksic, S., Uhitil, S., Petrak, T., Bazulic, D. and Karolyi, L.G., 2002. Occurrence of Vibrio spp. In sea fish, shrimps and bivalve molluscs harvested from Adriatic sea. *Food Control*, **13**, 491– 493

Jofre, J., 1992. Bivalve molluscs as vectors of human enteric viruses. *Microbiologie Aliments Nutrition*, **10**, 223–228

Mele, A., Stroffolini, T., Palombo, F., Gallo, G., Ragni, P., Balocchino, E., Tosti, M.E., Corona, R., Marzolini, A. and Moiraghi, A., SEIEVA Collaboratine Group., 1997. Incidence of and risk factors for hepatitis A in Italy: Public Health indications from a 10-year surveillance. *Journal of Hepatology*, **26**, 743–747

Proposal for a Regulation of the European Parliament and of the Council laying down specific hygiene rules for food of animal origin (consolidated draft) (2002), Council of European Union 11100/4/02 REV 4. Interinstitutional File 2000/0179 (COD)

Veterinary Research Communications, 28 (2004) 75–82

Veterinary Toxicovigilance: Objectives, Means and Organisation in France

G. Keck, P. Berny, F. Buronfosse, X. Pineau, E. Vermorel, B. Rebelle and T. Buronfosse
Centre National d'Informations Toxicologiques Vétérinaires, CNITV, Veterinary School of Lyon, Lyon, France
Correspondence: Centre National d'Informations Toxicologiques Vétérinaires, CNITV, Veterinary School of Lyon, BP 83, 69280 Marcy l'Etoile, Lyon, France
E-mail: g.keck@vet-lyon.fr

ORGANIZATION OF THE ANIMAL POISON CONTROL CENTER

The CNITV (Centre National d'Informations Toxicologiques Vétérinaires) at the National Veterinary School of Lyon has been operating as an Animal Poison Control Center since 1976 under the responsibility of the teachers from the Unit of Pharmacy Toxicology. It is organised as a non profit organization with the support of the fees from veterinary practitioners (about 650 adherents) and an executive board which defines the orientation and means of the center. It works in conjunction with the Center of Veterinary Pharmacovigilance and the Laboratory of Toxicological Analyses in the frame of the Unit of Pharmacy-Toxicology of the Veterinary School of Lyon. Since year 2000, the questions related to veterinary drugs (ADRs Adverse Drug Reactions) are dealt with by the Center of Veterinary Pharmacovigilance in the frame of the national system coordinated by the French Agency of Food Sanitary Security (AFSSA). So the CNITV deals with all questions related to veterinary toxicology except for veterinary drugs but including drugs for humans. The Laboratory of Toxicological Analyses performs various types of analyses on samples sent by veterinarians from the French territory, often after a contact with the Center. There are strong relations with the other disciplines of the Veterinary School, allowing for a rapid and quite efficient multidisciplinary approach of the cases and with the research activities in ecotoxicology of the department (bio-indicators, relation between biochemical toxicology and genetics).

MAIN OBJECTIVES

The main objectives of the CNITV are:

– to answer phone calls (24 h a day) or mail regarding any toxicologic question (diagnosis, treatment, prognosis evaluation, toxicological, pharmacological and environmental information on chemicals or noxious agents (plants, animals),

including differential diagnosis and help in case solving (samplings, contacts with specialists).
- to generate a field-cases database gathering all the questions, suspected or confirmed poisoning cases.
- to maintain scientifically accurate and up-to-date literature and manage a specific bibliographic database in order to analyze the cases recorded by phone or by mail.
- to participate in the initial and permanent education of veterinarians in the fields of toxicology (clinical, environmental).

In order to achieve its objectives, the Center has been developing various resources:

- human resources: a permanent veterinarian retributed by the center, a part time documentalist, a part time secretary, teachers and veterinarians acting benevolently, students of 2nd and 3rd cycle.
- computer databases: 3 specific systems: V-Tox is used to manage all the cases recorded by the Center (120,000 cases), Sentinel-vet® only deals with the management of veterinary drug related cases and Toxvet is concerned with the bibliographic references.
- a specific library of toxicology.

COLLABORATIONS

Collaborations are diverse:

- with other veterinary toxicological centers in France such as the one at the Veterinary School of Nantes or abroad (Pisa and Parma in Italy, IAPIC in the USA)
- with the veterinary professional organisations
- with human poison centers and the network of toxicovigilance
- with public or private organisations of animal welfare, food safety and environment protection
- with the industry of plant protection, animal feed.

GENERAL SURVEY

We will present here and discuss some statistical data describing the activity of the Center based on the present volume of cases (about 10,000 cases in year 2002 introduced in the database on a total number of 15,000 calls) and on the evolution observed in those recent years which shows a constant rise in the number of calls (from 4000 in 1991 to 15,000 in 2002) and the computerized cases.

Callers

About 75% of the calls come from veterinary practitioners. Territorial services (Laboratories and Veterinary Offices) represent about 5%, animal owners (pet owners, farmers) about 20%. Human Poison Centers call often, especially for cases concerning children exposed to veterinary products or pollution affecting together human and animal health.

Type of calls

(Fig. 1). Calls mostly are considered as emergencies or occur following an intoxication. In the first case the veterinarian is handling an animal which is, or may be, intoxicated and he asks about the treatment that should be instituted (60%). In the second case (27%), the problem previously occurred and the veterinarian wants to get more information about it (for example what samples to confirm the intoxication, what inquiry on the field). 8% of the calls ask for information on a product, often in

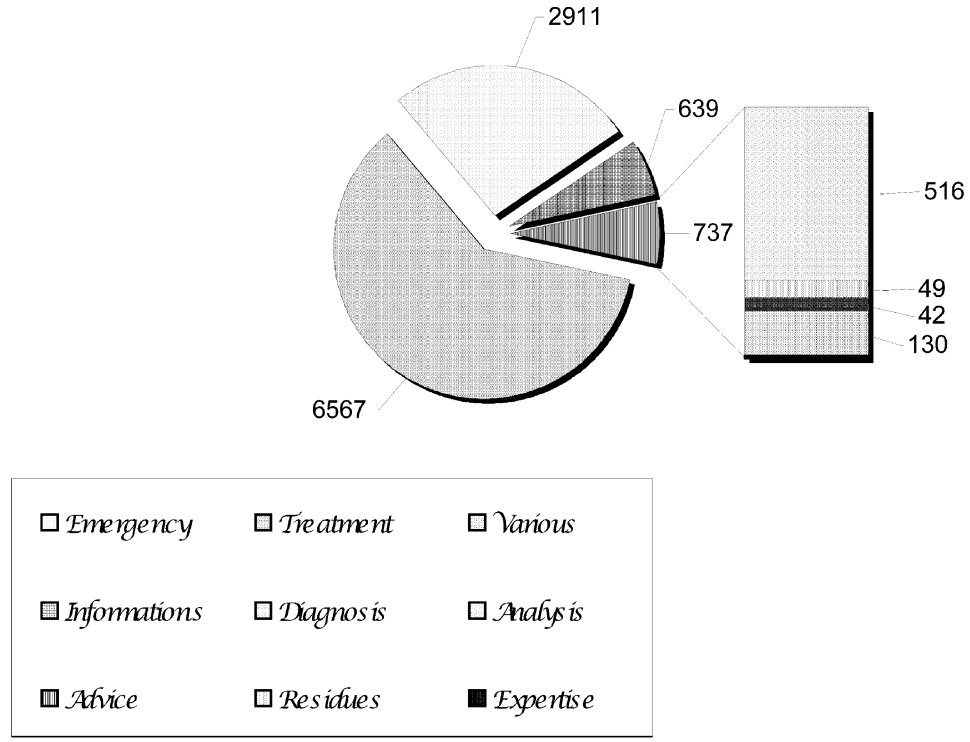


Figure 1. Motives for calls to the CNITV

order to prevent a risk of intoxication. Other questions concern residues or assistance in an expertise.

Species involved

(Fig. 2). Small animals represent the highest proportion, dogs mostly (68%) due to the important canine population and to their propensity to chew and swallow almost anything. Cats (18%), despite their natural mistrust, increase regularly in number of calls, due probably to the expanding medicalisation. The numbers of cases concerning new companion animals (rabbits, snakes) are sharply increasing.

Bovine (4%) and other farm animals represent a smaller percentage; despite the regular decrease in percentage, the number of calls is quite constant from one year to another. Besides animal health considerations, there are often concerns on chemical residues in animal products (milk, meat, eggs). Calls concerning humans are quite numerous (about 5%). Cases on wild terrestrial or aquatic animals are less numerous but often complex; they are often investigated by the laboratory.

Types of toxic agents involved

The presentation of the different toxic agents involved or suspected in the diverse animal species is not an easy task: there are obviously great differences of exposure and sensitivity between species; moreover, the causality assessment is often difficult. We developed for several years a probability scoring (or imputability assessment) of

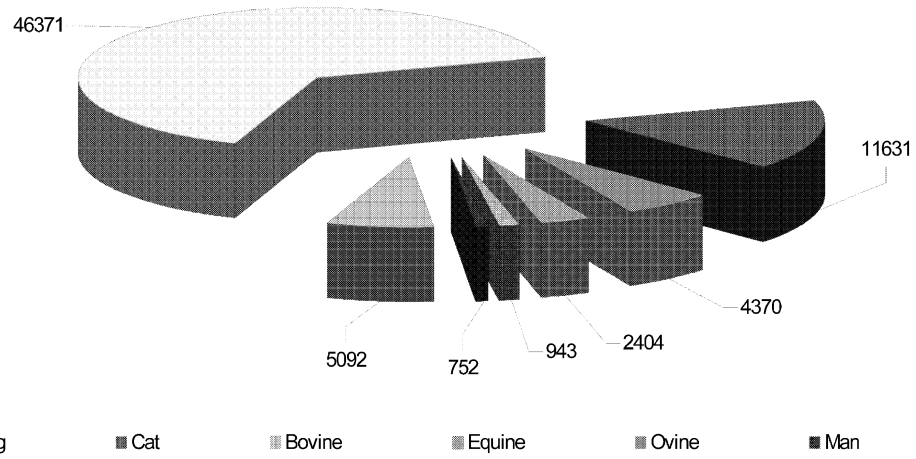


Figure 2. Involved animal species

the cases in 6 categories based on criteria of exposure, chronology and clinical features, sometimes laboratory analyses; this allows us to classify the cases as quite probably linked to the toxic agent (which represents approximately 30% of the calls, Droguet, 2002) or not. Still the causality assessment is not an "exact science" due to the uneven reliability of the data collected during or after the call and to the weak knowledge on the toxicity of many agents in the field conditions; it should regularly be reviewed on the basis of recent information. Quite often, a toxicological possibility is proposed by the Center on the basis of symptoms described, which obviously may lead to an uncertain assessment of the case.

For the different types of agents, the probability may be very unequal. For example, the implication of pesticides is less frequent than generally suspected by the callers. The current knowledge by the veterinarian of a toxicological issue may affect greatly the number of calls: for example, they call rarely for strychnine or metaldehyde intoxications or for toxic animals (snakes, caterpillars). So the statistical representation is certainly not to be considered as an exact picture of the intoxications in the field. However, the consideration of the data from the laboratory or specific surveys help to build a more exact representation.

Only large trends shall be presented here. Globally, the repartition between the principal groups of agents is indicated in Fig. 3.

Pesticides represent about 45% of the calls. The proportion is higher for herbivores than for carnivores and the different types of pesticides have various occurences in different species. Circumstances of intoxications are diverse; accidental via ingestion

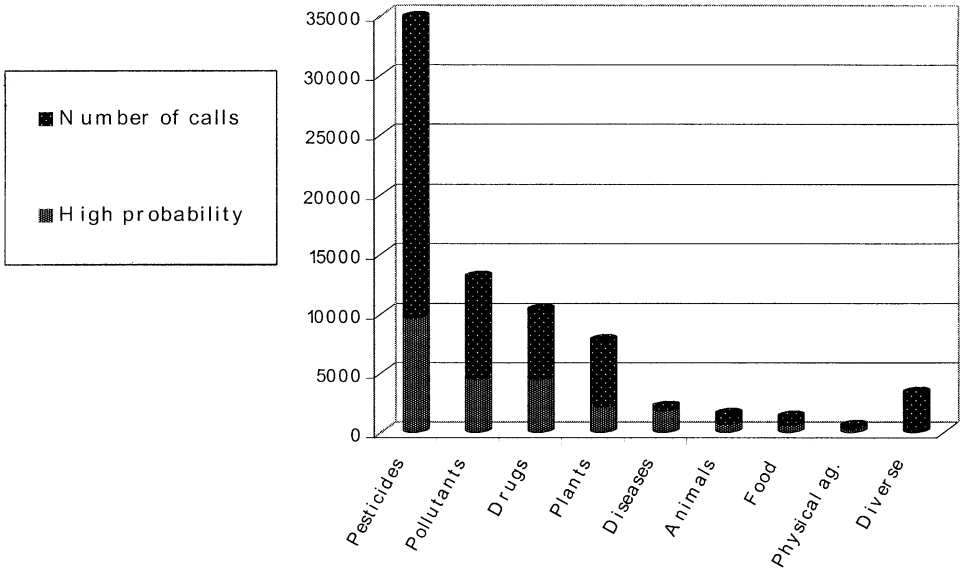


Figure 3. Repartition of calls among different categories of toxics

of badly stored products or water pollution, very often criminal. There were striking evolutions over recent years in the compounds involved linked to the suppression of many products such as nitrophenols, atrazine.

Insecticides cases (about 45% of pesticide calls) are dominated by organophosphates and carbamates, followed by pyrethroids. Herbicides are more frequently involved in cattle, although commonly used herbicides such as glyphosate are frequently encountered in small animals as well (Burgat *et al*, 1998). Rodenticides and molluscicides are very often involved in small animal intoxications (in about 50% of pesticides calls) especially antivitamins K (22% of pesticides calls) such as difenacoum and bromadiolone which appear most frequently. Old products are still frequently involved such as chloralose (4–5% of total small animal calls) or metaldehyde (about 1.5%).

Wild animals are often affected by pesticides as shown by the data from the center, but with a better accuracy from the laboratory. There are specific programs of surveillance on terrestrial fauna (Sagir program) and on fish.

The category "Pollutants" represents about 20% of the calls, with various types of compounds such as metallic or organic substances, household products, exposure to wastes etc.

Environmental pollutions more often concern production animals, especially bovines (lead, copper, dioxins, aromatic hydrocarbons, etc.); they often raise questions on residues in the animal products which implies their quantification and evaluation. A specific surveillance of cases related to exposure towards epuration waste recycling in agriculture (Ademe program).

Wild animals, especially fish, are also concerned; a special surveillance may be set up with environmental organisations.

Small animals are often affected by domestic products (detergents, disinfectants especially hypochlorite, paintings, anti freeze). Heavy exposure to petroleum is frequent in cats falling in a tank. There is an interesting correspondence with the risks observed in young children to the domestic chemical environment.

Plants represent around 11% of the calls. Small animals often ingest domestic plants (Ficus, Dieffenbachia) or garden plants (Laurel, Yucca). In cattle, predominant toxic plants are Taxus, Mercurialis, Galega especially in sheep, Amaranthus, acorn, bracken fern. Some plants are quite typical of a region such as Ferula in the South, especially Corsica.

Mycotoxins are often suspected especially in subacute episodes, but difficult to assess.

Toxic animals represent a great number of intoxications, essentially in dogs. But the number of cases registered is probably not representative, due to the good knowledge of the problem by the veterinarian. Toads, Vipera, venomous insects are the most frequently cited. Caterpillar Thaumatopea represents a major problem in the dog; it may also affect humans and shows an interesting geographical evolution from the South to the North of France.

Drugs

Up to year 2000, veterinary drugs were included in our statistics altogether with "human drugs", the total representing around 18% of the calls. Since 2001, the official system of veterinary pharmacovigilance implies a specific management of the cases concerning veterinary drugs (about 2000 cases in year 2001).

So toxicovigilance deals only with drugs for humans involved in domestic animals. The exposure may be via accidental ingestion of badly stored pills, which is very frequent in dogs with neuroleptics (especially benzodiazepines such as bromazepam), anti-inflammatory agents (ibuprofen), contraceptive pills. Obviously, the risks are very different for those different categories; countermeasures including antidotes were developed especially for diazepines intoxications (Bertini *et al*., 1995). Voluntary administration of drugs such as paracetamol or aspirin in cats, or loperamide in dogs of collie breed is another important source of drug toxicity, which may find interesting relations with pharmacovigilance.

Food and feeds

Pet food tolerance represents a growing concern with several cases registered. These are ususally quite difficult to assess and relate often to individual (or family) susceptibility. In dogs, chocolate ingestion is a common intoxication.

Production animal feed problems are probably underscored in our data because they are managed by nutritionists; they may relate with ionophore additives (monensin) for example in horses or in turkeys, but the incidence decreased due to an improvement in the incorporation procedures.

Diseases

In about 10% of the calls, the Center proposed an hypothesis of a non toxic disease on the basis of chronology and semiology. The caller was usually directed to a specialised laboratory to confirm the hypothesis.

CONCLUSION

The activities of a Veterinary Poison Center can initiate early help for diagnosis and appropriate treatment of animal intoxications. They also contribute to the initial and permanent education of veterinarians.

Careful analysis of the clinical and epidemiological experience is useful to determine current and future trends in field toxicology. This approach of toxicovigilance may also be used to investigate the impact of recently marketed chemical formulations

REFERENCES

Bertini, S., Buronfosse, F., Pineau, X., Berny, P. and Lorgue, G., 1995. Benzodiazepine poisoning in companion animals. *Veterinary Human Toxicology*, **37**(6), 559–562

Burgat, V., Keck, G., Guerre, P., Bigorre, V. and Pineau, X., 1998. Glyphosate toxicosis in domestic animals: a survey from the data of the Centre National d'Informations Toxicologiques Vétérinaires. *Veterinary Human Toxicology*, **40**(6), 363–367

Droguet, E., 2002. Les intoxications des animaux domestiques: bilan du CNITV 1999. *Thèse de doctorat vétérinaire*, Université Claude Bernard, Lyon, 93 p. + annexes

Hugnet, C., Cadore, J.L., Buronfosse, F., Pineau, X., Mathet, T. and Berny, P.J., 1996. Loperamide poisoning in the dog. *Veterinary Human Toxicology*, **38**(1), 31–33

Le Blaye, I., 1987. General survey of the activity of the National Veterinary Antipoison Center. *Veterinary Human Toxicology*, **29**(suppl. 2), 111–112

Vermorel, E., 2002. Les intoxications des carnivores domestiques par les médicaments humains. Etude épidémiologique et toxicologique d'après les données du CNITV de Lyon concernant les anti-inflammatoires non stéroïdiens, le paracétamol et les benzodiazépines. *Thèse de doctorat en pharmacie*, Université Claude Bernard, Lyon, 142 p. + annexes

Veterinary Research Communications, 28 (2004) 83–88

Incidence of Poisonings in Domestic Carnivores in Italy

A. Giuliano Albo and C. Nebbia*
Pharmacology and Toxicology Unit, Department of Animal Pathology, University of Turin, Via Leonardo da Vinci, 44, 10095 Grugliasco (TO), Italy
**Correspondence: Dipartimento di Patologia animale, Università di Torino, Via Leonardo da Vinci, 44, 10095 Grugliasco (TO), Italy*
E-mail: carlo.nebbia@unito.it

Keywords: poison centre, cat, dog, toxicosis, epidemiology

INTRODUCTION

The Italian Veterinary Toxicologic Assistance Service (Servizio di Assistenza Tossicologica Veterinaria, S.A.T.V.) was established in mid 1996 at Turin. It differs from other European Poison Centres in that it has been promoted and is operated by a cultural association of veterinarians devoted to small animal practice (Società Culturale Italiana Veterinari Animali da Compagnia, S.C.I.V.A.C.) under the scientific advice of the Pharmacology and Toxicology Unit of the Department of Animal Pathology of the University of Turin. Other special characteristics of the Service are that it may be accessed only by licensed veterinarians and is completely free of charge. Of course, this means that it needs to be sponsored and given grants by private companies. The main aims of S.A.T.V. are the following:

- to collect relevant toxicological information from different sources (assessed field cases, scientific literature) and to store it in a computerised database;
- to provide real-time telephonic assistance to colleagues facing toxicological emergencies in dogs and cats;
- to perform epidemiological studies based on the telephone enquiries;
- to promote the exchange of information with other national or international Poison Centres.

The S.A.T.V. is active 24 h a day; the staff is made up of group 12 colleagues with special training in veterinary clinical toxicology who have access to a dedicated computerized database which is periodically updated on behalf of the staff of the Pharmacology and Toxicology Unit of the Department of Animal Pathology of the University of Turin. Xenobiotics have been divided into the following classes: veterinary and human drugs, agrochemicals (insecticides, herbicides, fungicides), rodenticides, molluscacides, miscellaneous, halogens, metals and antidotes. Each file bears the commercial trade names of drugs, pesticides or products containing the active principle(s), lethal and/or toxic doses for both dogs and cats (or laboratory animals), clinical signs, clinicopathology, laboratory diagnosis and antidotal and/or supportive

treatment. Phone calls are recorded and stored in the database and a file is automatically generated for subsequent retrieval containing information regarding the (geographical) region of the caller, the species involved (cat or dog), as well as the age, sex, breed and environment (urban or rural) of the affected animal. The present work shows all results collected from the SATV from May 1996 to May 2003. It is necessary to specify that calls are stored in the database only when they are not too generic and refer to a known active principle. Calls for information queries have not been considered in the present report.

RESULTS

Since May 1996 the SATV has stored 4297 calls coming from all over Italy on its data base. Calls come especially from the North (66%), probably because of a better knowledge of the service by veterinary practitioners. Enquiries from Central and Southern Italy make up the remainder of calls (34%). The SATV has mostly been contacted for problems involving dogs (80% of total calls). The lower incidence of calls concerning cats may be attributed to the more "prudent" feeding habits characterising this species together with its tendency to stay by itself when feeling "sick": this happens especially for individuals living in country areas or in a semi-domestic state. According to our data, poisonings appear to be relatively more frequent in urban (57%) than in rural areas, not only because of the large array of potentially toxic household products, but also because poisonings affecting animals living in a restricted area such as a flat are more easily detected.

A breakdown of all enquiries by class of chemicals is depicted in Table I. Veterinary and, to a far lesser extent, human drugs are responsible for the greatest percentage of calls in both species: namely 23% for dogs and 35% for cats. Within this class, antiparasitic compounds account for (as many as) 64% and 79% of the calls in dogs and cats, respectively, followed by non steroidal antiinflammatory agents (10% for dogs and 7% for cats). The majority of the enquiries concerning antiparasitic drugs refer to principles active against external parasites, such as pyrethroids and organophosphorus compounds or carbamates. In most cases misuse by the animal's owners or the accidental ingestion of commercial preparations such as collars, powders or sprays containing such compounds has been the cause of poisoning. In this respect it is worth noting that, on a percentage basis, pyrethroids (mostly permethrin) account for nearly half of the calls concerning this subclass in the cat, thereby confirming the particular sensitivity of this species toward these derivatives (Martin and Campbell, 2000). According to our data, the improper use of ivermectin (49 calls) may still represent a problem not only for Collie dogs, which are reported to be most susceptible to this drug but also for other dog breeds as well as cats (Nelson *et al*., 2003). Chlorpyriphos (47 calls for dogs and 12 for cats), Amitraz (38 calls for dogs), and Fipronil (28 calls for dogs and 21 for cats) are the subject of a significant number of inquiries; the latter is normally very well tolerated but multiple applications of very

TABLE I
Calls stored from SATV (May 1996 – May 2003)

Class	Dog	Cat
DRUGS	**773 (23%)**	**321 (35%)**
Antiparasitic	494 (64%)	254 (79%)
Organophosphorus (*Chlorpyiriphos*)	143 (27%)	43 (18%)
Carbamates (*Propoxur*)	62 (9%)	17 (7%)
Pyrethrines-pyrethroids (*Permethrin*)	122 (27%)	126 (27%)
Others (*Ivermectin, Amitraz, Fipronil*)	167 (37%)	68 (26%)
Antiinflammatory agents (*Ibuprofen, Paracetamol*)	77 (10%)	21 (7%)
Psychoactive drugs (*Hashish, Cannabis indica*)	30 (4%)	1 (<1%)
Hormones (*Oestrogens*)	32 (4%)	3(1%)
Others (*Chemo-antibiotics*)	140 (18%)	42 (13%)
AGROCHEMICALS	**650 (19%)**	**124 (14%)**
Insecticides	267 (40%)	65 (55%)
Organophosphorus (*Diazinon, Malathion*)	83 (31%)	16 (25%)
Carbamates (*Carbaryl, Methomyl*)	85 (32%9	12 (18%)
Pyrethrines-pyrethroids (*Permethrin*)	28 (10%)	19 (29%)
Organochlorine (*Endosulfan*)	58 (22%)	13 (20%)
Others	13 (5%)	5 (8%)
Fungicides (*Copper sulphate*)	173 (27%)	31 (25%)
Fertilizers (*Nitrogen derivatives*)	43 (7%)	4 (3%)
Herbicides (*Paraquat, Glyphosate*)	167 (26%)	24 (17%)
MISCELLANEOUS	**668 (20%)**	**224 (25%)**
Household products (*Sodium hypochlorite*)	147 (23%)	57 (25%)
Hydrocarbons (*Diesel oil*)	41 (6%)	52 (23%)
Zootoxins (*Snake venoms*)	129 (19%)	7 (3%)
Others (*Camphor, glue*)	55 (17%)	9 (4%)
RODENTICIDES	**647 (19%)**	**48 (5%)**
Anticoagulant	495 (77%)	28 (52%)
I generation (*Racumin/Coumatetrarhyl*)	157 (32%)	6 (21%)
II generation (*Bromadiolone, Bromadifocoum*)	285 (57%)	18 (65)
Indandion derivatives (*Chlorphacinone*)	53 (11%)	4 (14%)
Others (*Zinc phosphide, Strychnine*)	152 (22%)	20 (48%)

Continued

high dosages (particularly to puppies and kittens) may give rise to nervous stimulation which is related to the blocking of GABA receptors brought about by this drug (Hainzl *et al*., 1998). As regards the NSAIDs, ibuprofen, paracetamol, and aspirin account for the majority of the inquiries for dogs, and paracetamol and aspirin for cats, which are highly sensitive to these drugs. In most cases animals were poisoned

TABLE I
Continued

Class	Dog	Cat
DRUGS	**773 (23%)**	**321 (35%)**
MOLLUSCICIDES	**286 (8%)**	**31 (3%)**
Metaldehyde	183 (64%)	26 (84%)
Methiocarb	103 (36%)	5 (16%)
PLANTS	**163 (5%)**	**99 (11%)**
House plants (*Euphorbia pulcherrima, Ficus spp.*)	64 (40%)	73 (70%)
Others (*Nerium oleander, Nicotiana tabacum*)	99 (60%)	26 (30%)
METALS (*Lead, mercury*)	**131 (4%)**	**32 (4%)**
OTHERS	**76 (<3%)**	**24 (<3%)**

Note: in brackets are reported the most commonly involved active principles included in the related class or subclass.

as the result of the accidental ingestion of tablets intended for human therapy. Finally, hormones and psychoactive drugs are implicated in a small percentage of calls in dogs (4%), the former class being entirely represented by oestrogen-based oral contraceptives, the latter by hashish (15 calls) and *Cannabis indica* (11 calls).

Agrochemicals are responsible for a significant percentage of enquiries for both species (19% of the total calls for dogs, 14% for cats). Insecticides are the most represented class (40% for the dog, 55% for the cat). For dogs the most implicated compounds are carbamates, especially Methomyl (39 calls) and Carbaryl (22 calls), organophosphorus compounds with particular regard to Diazinon (28 calls) and Malathion (21 calls), and organochlorine compounds, with 31 calls regarding Endosulfan. For both species, fungicides account for nearly one-fourth of the calls of the agrochemical class and the majority of these (43% for dogs and 52% for cats) are related to the accidental ingestion of copper sulphate. As regards herbicides, the most represented active principles for both species are Paraquat (47 calls for dogs and 5 for cats), mostly as the result of malicious poisonings, and Glyphosate (85 calls for dogs and 14 for cats), which is usually reported to affect animals ingesting grass and vegetation recently sprayed with a glyphosate-based commercial preparation. In this respect it should be noted that a surfactant present in many liquid preparations may be responsible for most of the reported toxic effects, including vomiting, diarrhoea, and nervous symptoms (Burgat *et al*., 1998).

Miscellaneous agents account for one-fifth of the calls in both species (20% in dogs and 23% in cats). The high percentage of enquiries is probably due to the great number of compounds included in this class (household products, solvents, hydrocarbons, zootoxins, and so on). It is important to stress the considerable role played

by zootoxins (136 calls), particularly as regards venomous bites by *Vipera* spp.; over 95% of these cases involve dogs. Household products (particularly sodium hypochlorite and detergents) are also responsible for a large number of enquiries, thereby confirming that companion animals (like human beings) may be daily exposed to serious dangers even in a relatively "safe" environment like a flat. In addition, our centre has received 20 enquiries regarding exposure to petroleum distillates (petrol, diesel, etc.) in cats, which are more prone to ingest considerable amounts of these derivatives through grooming.

Rodenticides represent another class of xenobiotics heavily involved in pet toxicoses (19% of the total calls for dogs and 5% for cats). The higher incidence displayed by the canine species over the feline one is most likely attributable to the less selective feeding habits of dogs coupled with the malicious use of these compounds as baits. Anticoagulants account for the majority of these calls, especially in dogs (77%), with an extremely significant role played by the so called "second generation" molecules, particularly Bromadiolone (109 calls for dogs and 11 for cats) and Brodifacoum (89 calls for dogs and 4 for cats), which are far more dangerous than those belonging to the "first generation" (e.g. Warfarin) because they may cause fatal poisonings even after a single exposure (Petrus and Henik, 1999). Older but still largely employed rodenticides like zinc phosphide (52 calls) and strychnine (39 calls) still represent a considerable threat to dogs.

Despite the relatively low incidence of enquiries concerning molluscicides (8% and 3% of the total calls for dogs and cats, respectively), this class includes very toxic active principles like Metaldehyde and Methiocarb (Puyt, 1995). Indeed, Metaldehyde represents the first cause of enquiries for dogs (183) and a significant number of calls (103) were also concerned with Methiocarb, suggesting the low efficacy of the repellents that are usually added to commercial preparations.

As far as toxic plants are concerned, our data point to a greater sensitivity of the cat (11%) as compared to the dog (5%). Unlike dogs, felines seem to be more prone to be poisoned by house- or ornamental plants, which are involved in nearly 70% of all enquiries related to phytotoxins, with *Euphorbia pulcherrima* (Poinsettia) and *Ficus* spp. accounting for the majority of the calls. According to our data, dogs are more frequently affected by wild plants (60% of the calls in this class), although a significant number of cases have involved the accidental ingestion of tobacco.

Finally, metals (especially lead and iron salts) no longer seem to be a primary cause of poisoning in domestic carnivores, representing 4% of the total enquiries for both species. Interestingly, mercury (35 calls) accounts for more than one third of the calls pertaining to this class in the dog as the result of the accidental ingestion of thermometers, although the metal content is normally too low to pose relevant clinical problems.

DISCUSSION AND CONCLUSIONS

When considering the total number of stored calls, it should be stressed that, unlike similar Centres, the work of S.A.T.V. is currently restricted to cases of poisoning

concerning domestic carnivores and the team is not allowed to address enquiries from non licensed veterinarians. In addition, the Centre is not yet equipped with its own facilities for performing the toxicological analyses.

As regards the reliability of the generated epidemiologic data, one should take into account that the practitioner is usually more likely to contact the Centre when facing a case involving a "new" or an unusual poison rather than a known active principle. This may at least partly explain the relatively low percentage of the calls concerned with "classic" poisons such as for instance strychnine, Paraquat, organophosphorus or lead and perhaps the relatively high frequency of the enquiries about (some) "emerging" poisons such as Glyphosate, Methomyl and Endosulfan. Nonetheless, the collected data indicate how dangerous the misuse of the veterinary drugs can be (in particular that of external antiparasitic agents), since they represent as many as one third and one fourth of the total calls for cats and dogs, respectively. The potential danger of certain agrochemicals and of many cleaning products also has to be stressed. Finally, one should also emphasize the high incidence of calls related to second generation anticoagulant rodenticides. Since most cases arise from the ingestion of baits, in our opinion the free sale of commercial preparations containing these very toxic active principles should be restricted or even no longer be allowed.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Federica Gusson, Dr. C. Scotti and Sig. Gianluca Maggiora. The work was partly supported by grants from Nestlé Purina PetCare Italia S.p.A.

REFERENCES

Burgat, V., Keck, G., Guerre, P., Bigorre, V. and Pineau, X., 1998. Glyphosate toxicosis in domestic animals: a survey from the data of the Centre National d'informations Toxicologiques Vétérinaire, *Veterinary and Human Toxicology*, **40**(6), 363–367

Hainzl, D., Cole, L.M. and Casida, J.E., 1998. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct, *Chemical Research in Toxicology,* **11**(12), 1529–1535

Nelson, O.L, Carsten, E, Bentjen, S.A. and Mealey, K.L., 2003. Ivermectin toxicity in an Australian Shepherd dog with the MDR1 mutation associated with ivermectin sensitivity in Collies. *Journal of Veterinary Internal Medicine,* **17**(3), 354–356

Martin, A. and Campbell, A., 2000. Permethrin toxicity in cats. *Journal of Toxicology – Clinical Toxicology*, **38**(2), 95–101

Petrus, D.J. and Henik, R.A., 1999. Pericardial effusion and cardiac tamponade secondary to brodifacoum toxicosis in a dog, *Journal of the American Veterinary Medical Association*, **215**(5), 47–648

Puyt, J.D., 1995. Sémiologie et diagnostic différentiel des intoxications par les convulsivants chez les carnivores domestiques, *Recueil de Médecine Vétérinaire*, **171**, 109–114.

Veterinary Research Communications, 28 (2004) 89–95

Epidemiology of Intoxications in Italy

M. Amorena*[1], F. Caloni[2] and G. Mengozzi[3]
[1] *Department of Food Science – Faculty of Veterinary Medicine – University of Teramo;* [2] *Department of Veterinary Science and Technologies for Food Safety – Faculty of Veterinary Medicine, University of Mila;* [3] *Department of Veterinary Clinic, Faculty of Veterinary Medicine, University of Pisa, Italy*
* *Correspondence: Department of Food Science, Faculty of Veterinary Medicine, University of Teramo, Piazza Aldo Moro ,64100, Teramo, Italy*
E-mail: amorena@unite.it

Keywords: poisonings, domestic animals, Italy

INTRODUCTION

In contrast with France, where the Centre National d'Informations Toxicologiques Veterinaires (CNIVT), has been operating for several years to prevent poisoning, giving information about treatments, performing toxicological analysis and, above all, giving epidemiological predictions to vets, associations and/or citizens, Italy has not yet developed such a structure able to coordinate knowledge in the toxicological field. So, to date, in our country, incidence of poisoning in domestic animals has only been partially evaluated in isolated studies. This deficiency is reflected by the exact knowledge of these phenomena. In fact, in Italy the incidence of poisonings in domestic animals has only been partially evaluated through isolated studies and the data reported are not representative of reality as they generally refer only to analytical results obtained from IIZZSS, from Toxicological Veterinary laboratories of Faculties of Veterinary Medicine or referred to on-phone surveys results (SATV). Moreover, these studies particularly consider Central and Northern Italy, and the data relating to Southern Italy are scarce.

Therefore, there is a critical lack of information for practitioners in this field and a real risk of misevaluating this important phenomenon. So, the present paper tries to define, in a better way, the incidence of poisonings in companion animals in Italy and to improve knowledge of this phenomenon.

The presentation is divided in three parts. In the first one F. Caloni presents the situation in the Northern Italy with the survey of the Poison Control Centre of Milan (CAV) from 2000 to 2002. In the second part G. Mengozzi reports the results from 1999 to 30 June 2003 of Central Italy; and in the last one M. Amorena shows the incidence of poisonings in Southern and Insular Italy and for this purpose were created survey cards which were proposed to practitioners through on-phone survey.

EPIDEMIOLOGICAL STUDY OF POISONINGS IN DOMESTIC ANIMALS IN NORTHERN ITALY

The Poison Control Centre of Milan (CAV) Niguarda Hospital, was established in 1967 and receives 64% of the total national requests for toxicological consulting each year from all Poisoning Centres active in Italy.

In the period between January 2000 and December 2002, our active collaboration permitted the recording and classification of data, and also a "follow up" of the veterinary cases. Our epidemiological investigation takes into consideration animal intoxications from Northern Italy, considering a total of 342 contacts.

34.7% of the calls received by CAV were from owners of animals and 65.3% from vets. 90.5% of the total consulting in the CAV was related to dogs and cats, and only a low percentage related to other species like bovine, equine, birds, rabbits, swine, ovine and exotics. 55% of dogs and cats had an age of less than 2 years, 34% from 2 to 8 years, and 11% over 8 years. 43% of the sample were males while 57% were females.

For the categories of toxics responsible for poisoning pesticides are the highest class, followed by household products, drugs, plants, industrial products and animal poisonings. The class "other" includes abuse substances, food, and cosmetics. Rodenticides (36%) are most responsible for intoxication in the class of pesticides, followed by insecticides (25.6%), herbicides (24.3%), fungicides (8.1%) and molluscicides (6%). The "households" class consists of detergents, but also batteries, solvents and thermometers. Industrial chemicals including fuels, heavy metals and ethylene glycol represents the causative agent most involved in the dog. 74% of the cases of the toxic agent happen in the domestic environment, either in the house or outside (garden). 13.5% of poisonings happen outside the domestic environment (open space). Only a small percentage of intoxications have been verified in the place of work or in public places. Respect to the frequency of exposure, 71% of the subjects came into contact with the toxin only once, 3.5 more than once, and the frequency was unknown for the remaining 25.5%. The occurrence reliability, that is the assurance of the owner of exposure to the toxin, was certain for 51.4% of cases, probable for 27.3% and unknown for 21.3% of the intoxications. The cause of intoxication was accidental for 81.92%, iatrogenic for 3.32%, fraudulent for 2.58% and unknown for 12.18% of cases. The most frequent route of intoxication was by ingestion and was not reported on the schedule for 6.9%. 52% of the subjects were not tested by laboratory analysis; and we did not receive information for 29.5%. The result was reported for 46.8% of cases, with recovery for 42%, permanent disability for 1.3% and in 3.5% of the cases the animals died. The toxicological risk was assessed as "high" for 14.5%, "medium" for 16%, "low" for 8.2%, "not excluded" 28%, "excluded" 10.6%, while for 22.7% this was not assigned. Comparing the incidence of the type of poisoning in this investigation with the previous survey, where pesticides were the most frequent poisoning class, it has been observed that this is now the rodenticides. From the analyzed data it appears that zinc phosphide is a frequent causative agent of intoxication in

dogs. Poisoning by plants is also increased as compared with the last survey of the CAV. Intoxication by plants took place in the garden, and oleander was the most responsible plant, in the flat *Euphorbia Pulcherrima* was the most involved, once again in dogs. Under the class "drugs" we intend only products for human use, imprudently left by the owner within reach of animals. Some of these intoxications showed a seasonal trend, like the increase in intoxications by antihistaminic drugs in spring. The comparison between dogs and cats demonstrates that for the cat the domestic environment, in the house, is the place were intoxications most easily happen (62.5%). This could be related to the life-style of these animals. In Italy cats living in urban areas normally do not have the possibility to go outside from the house. The most frequent route of exposure to toxins is by ingestion. It is very important to underline that for 53.2% of cases the result of the intoxications remained unknown, despite our strict follow-up. We can conclude that our investigation for 2000–2002 related to the intoxications in Northern Italy, gave important and useful results for operators and demonstrated the utility of ongoing collaboration with the Poison Control Center, University, Veterinarians, and other organizations to give rapid and efficient therapeutic aid, and also to create a up-to-date database of intoxications in domestic animals.

EPIDEMIOLOGICAL STUDY OF POISONINGS IN DOMESTIC ANIMALS IN CENTRAL ITALY

Since 1996, when it was founded, the Laboratory of Toxicology of the Veterinary Clinic Department (University of Pisa) has carried out a service of veterinary toxicological diagnostics and annually receives many requests for consultancy from self-employed veterinarians, government corporations and private citizens, regarding suspicious individual or general poisonings in domestic or wild animals.

In the period from 1 January 1999 to 30 June 2003 the Laboratory received 746 samples for a total of 1248 requests for analyses, 492 of which were found to be positive (40%). Based on clinical symptoms or anatomo-pathological exam, the veterinary or Laboratory personnel suggest looking for one, two or more compounds, suspected to be the cause of the toxicological event, for a medium value of 1.7 analyses of samples.

The number of different toxic substances for which analysis has been requested and the percentage of those which really resulted positive from 1999 to the present have revealed how cholinesterase inhibitors play a role of primary importance. As a matter of fact, these substances are easily available in many formulations and it is possible to buy them without any particular restriction. Rodenticide poisonings caused by anticoagulants are less frequent; however they still show a high positivity. With these accidental poisoning seems more probable, due to struggles against rodents. As regards the frequency of poisoning due to zinc phosphide, its incidence is not particularly high and remains the same during the entire period taken into consideration.

Metaldehyde poisoning (percentage of positivity of 11% in 1999) has increased over the years, reaching 31% in the first six months of 2003. Metaldehyde is a much-used molluscicide and is easily available, whose use has increased to prepare poisoned baits. A particular case is represented by strychnine which is known as a very dangerous poison widely used in the 80s: this now justifies the high number of requests. However, its employment is nowadays under strict legal restriction and limits its real effects on the total number of analyses found positive (Table I).

Investigation of the positive results for toxicants examined obtained for biological material from different species has underlined the definite predominance of the dog as the animal species that risks intoxication the most. In second position, considerably less at risk, is the cat, followed by avian species, the horse, the fox and cattle. One of the most important causes of the high frequency of intoxication in the dog is its tendency to eat everything around it, unlike the cat which is extremely suspicious towards any unfamiliar compound. Another important source of poisoning in the dog is malicious poisoning, which seems to be related to the hunting season when animals are taken to areas where they are not welcome. The low number of reports for the horse and cattle is probably connected to their conditions of life: there are few occasions that permit these animals to feed themselves freely and without controlled fodder.

It is important to note the high percentage of positivity found for baits discovered by chance (74%), for which it has been possible to find a remarkable heterogeneity of preparation, always characterized by the presence of elevated concentrations of toxin. The substances mainly used in these formulations are cholinesterase inhibitors (organophosphorates and carbamates), whose high toxicity makes them particularly suitable for malicious employment.

The differences in the incidence of positive samples in the different periods of the year deserves particular attention. It is in fact possible to note how, uniformly for the four years considered, the poisonings are more frequent during spring (from March to June), during which the number of animals living in the open air increases and,

TABLE I
Number of requested analyses and incidence of positivity (%) in the period 1999/2003

	1999	2000	2001	2002	2003 (1° sem)
Cholinesterase inhibitors	110 (67%)	102 (39%)	124 (50%)	126 (62%)	77 (40%)
Anticoagulant rodenticides	29 (55%)	67 (60%)	34 (29%)	24 (42%)	24 (67%)
Strychnine	37 (16%)	35 (14%)	54 (17%)	51 (14%)	40 (5%)
Zinc phosphide	15 (47%)	23 (30%)	27 (33%)	15 (33%)	12 (25%)
Metaldehyde	9 (11%)	25 (16%)	31 (35%)	50 (28%)	49 (31%)
Others	10 (10%)	12 (17%)	7 (14%)	17 (35%)	12 (0%)
TOTAL	210	264	277	283	214

consequently, the people annoyed by them. Even intoxications in the month of September are very frequent, coinciding with the opening of the hunting season. In this period poisoned baits are usually spread to exterminate the natural predators of the game (foxes etc) or to damage competitors. The low values obtained for the months of December and January are due to the fact that in this period animals live mostly indoors and are therefore less subject to intoxications.

Finally, it is important to underline that some malicious poisonings are very dangerous even for humans, who can easily come into contact with toxic substances used in baits spread in public and private gardens.

EPIDEMIOLOGICAL STUDY OF POISONINGS IN DOMESTIC ANIMALS IN SOUTHERN AND INSULAR ITALY

The aim of this study was to provide poisonings information for dogs and cats in Southern and Insular Italy and to determine the poisons more frequently involved. For this purpose were created survey cards which were proposed to practitioners through on-phone survey. The regions considered are: Abruzzo, Molise, Lazio, Puglia, Campania, Calabria, Basilicata, Sicily and Sardinia. For the Abruzzo Region it was possible to correlate data obtained through on-phone survey with the analytical results of toxicological analysis performed by the Istituto Zooprofilattico dell'Abruzzo e del Molise "G. Caporale".

63.2% of 310 practitioners contributed to the survey by answering our questionnaire. The highest interest and participation in the survey was observed in the regions of Abruzzo, Molise and Lazio with a percentage of 79.1%, 87.5% and 82.1%, respectively. The total number of poisonings considered were 17,324; 12,012 concerning dogs and 5,312 for cats. 45.1% of cases were suspected to be intentional. Only 56.1% of the veterinarians interviewed sent material to confirm their diagnosis to a laboratory. For pets that were still alive (81% of cases), a blood sample was the material preferably sent to perform hematochemical and coagulative evaluation. In 48.6% of cases, the gastric content was analysed, while urine and faeces analyses occurred respectively in 16.2% and 10.6% of cases. For dead animals or those that died during hospitalisation, 54.3% of vets sent the whole carcass to the lab, while the others performed an autopsy and sent organs or tissues to the laboratory for specific toxicological analysis. Some practitioners related that they usually contact the Anti-poisoning Centre of Torino (SATV) to have phone-support for diagnosis. As regards the classes of poison implicated, 84% of cases were related to anticoagulants (AC) and Organophosphate (OP), while 7.6% and 6.2% were associated to metaldehyde (MET) and strychnine (STR), respectively. Other toxic substances causing poisonings were also signalled but with an incidence less than 1%.

As reported in Table II, the high number of requests for specific toxicological analysis is closely related to a wider range of toxic compounds found. Particularly in Abruzzo-Molise and Lazio where 60% of vets sent samples to a lab, the number of

TABLE II
Percentage of vets who sent samples to a laboratory to request specific toxicological analysis for toxic compounds causing poisoning in companion animals. The regions considered are: Abruzzo (A), Molise (M), Lazio (L), Puglia (P), Campania (Camp), Calabria (Cal), Basilicata (B), Sicily (Sic) and Sardinia (Sar)

	A (%)	M (%)	L (%)	Cam (%)	Cal (%)	P (%)	B (%)	Sic (%)	Sar
Lab. Refer.	85.3	71.4	63	50	53.8	46.4	50	35.3	9.1
OP	26.5	19.7	23.3	28.5	39.6	34.3	39.3	49.2	44.5
AC	27.5	60	32.1	64.2	53.4	56.6	54.7	44.1	30
MET	15.3	8.3	19	2.2	2.3	7.1	—	—	14.5
STR	13.3	12	15.3	1.7	3.9	0.2	2.4	2.4	4.5
OC	4.1	—	2.1	—	—	—	—	—	2.3
CA	2	—	1.4	1.9	0.8	0.5	0.7	1.8	1.8
Tox plant	2	—	—	—	—	—	—	—	—
Ethylene gly.	3.1	—	2.1	—	—	0.3	1.4	0.3	0.4
Viper bite	3.1	—	0.7	—	—	—	—	—	—
Urea	1	—	0.7	—	—	—	—	—	—
Copper sulphate	1	—	0.7	—	—	—	—	—	—
Naphtha	1	—	—	—	—	—	—	—	—
Lead	—	—	0.7	—	—	—	—	—	—
Arsenicals	—	—	1.4	—	—	—	—	—	—
Paraquat	—	—	—	—	—	1.3	—	—	—
Toluene	—	—	—	—	—	—	—	—	0.9

toxic substances revealed were 12 versus 7 for the other regions of southern Italy. The average percenrage of vets requesting specific analysis in the other southern regions was about 50%, falling to 35.3% for Sicily and 9.1% for Sardinia. Such results are probably related to the lack of specific laboratories in these areas or to the tendency of vets to use only their own experience to perform diagnoses. However, it is important to note that many toxic substances produce similar neurological symptoms, highlighting the very real importance of specific analysis in differentiation of causes of poisoning. Without analysis, there is a particularly frequent confusion between OP poisoning and other neurotoxic substances, which are, thus, less frequently signalled and revealed. While we can observe a seasonal incidence for OP poisonings, for AC, MET and STR poisoning this was not observed. Even if MET is currently used in agriculture during spring and autumn, the constant presence and referral of MET poisoning is related to bait frequently prepared to criminally poison dogs and cats. We also found a correlation between agricultural practices and incidence of some poisonings. Particularly in Puglia the diffusion of permanent cultivation justifies the 1.3% of paraquat poisoning observed. Conversely, less intensive soil utilization in Molise is in accordance with the low frequency of OP poisoning. In Sicily, where

permanent cultivation is widely practised, OP poisoning is very frequent, representing the highest percentage observed (48.2%).

For Abruzzo region, following previous studies, we were able to benefit from the data from the lab of the Istituto Zooprofilattico dell'Abruzzo e del Molise "G. Caporale". Toxic substances more often reported by vets were AC and OP while other compounds were reported less. These data confirm the trend observed in a previous work performed during the period 1997–2000 in the Abruzzo region where the percentages of poisonings signalled were 79.2% for AC, 69.4% for OP, 38.9% for MET and 37.5% for STR. Interestingly it can be observed that only 0.8% of samples were positive for AC. This result is probably related to the ability of veterinarians to diagnose AC poisoning directly from anamnesis and clinical symptoms, without the necessity to refer to the lab, while for the other compounds the veterinarians usually sent samples to IZS to confirm the poisoning.

These data are similar to those obtained during the period 1999–2001 in the Tuscany region and reported by other authors indicating a similar trend. In contrast, considering the data obtained in a study performed in Friulia Venezia Giulia during the period 1997–2000 by IZV delle Venezie and the University of Padua, the compounds most commonly identified were plant protection products (49%): particularly OC, while OP were found in 21% only of cases. STR was also an important cause of poisoning in Friuli Venezia Giulia while AC and MET seemed to be less important.

REFERENCES

Amorena, M., Calzetta, L., Ruggeri, E., Ricci, A., Cavaliere, M. and Crescenzo, G., 2001. Avvelenamenti nel cane nella regione Abruzzo nel quadriennio 1997–2000. *Atti Società Italiana di Scienze Veterinarie* LV, 303–304

Binato, G., Benetti, C., Piro, R., Boancotto, G. and De Liguoro, M., 2002. Avvelenamenti negli animali: casistica riscontrata dall'IZS delle Venezie nel quadriennio 1997–2000. *Atti Società Italiana di Scienze Veterinarie*, LVI, 237–238

Caloni, F., Faraoni, L., Bacis, G., Zoppi, F., Virzì, L., Fracchiolla, M.L., Pompa, G. and Davanzo, F., 1991. Intossicazioni veterinarie: casistiche del Centro Antiveleni di Milano anni 1991–2000. *Atti della Società Italiana delle Scienze Veterinarie*, LV, 301–302

Calzetta, L., Cavaliere, M., Ruggeri, E., Ricci, A., Crescenzo, G. and Amorena, M., 2002. Incidence of intentional poisoning of dogs in the Abruzzo Region of Italy. *Veterinary and Human Toxicology*, **44**, 111–113

Giorgi, M., Meucci, V., Loni, R., Soldani, G. and Mengozzi, G., 2002. Intossicazioni in medicina veterinaria. *Obiettivi & Documenti Veterinari*, **6**, 57–62

Hornfeldt, C.S. and Murphy, M.J., 1997. Poisonings in animals: the 1993–1994 report of the American Association of Poison Control Centers. *Veterinary and Human Toxicology*, **39**, 361–365

Lorgue, G., Lechenet, J. and Rivière, A., 1999. *Tossicologia Clinica Veterinaria*, Cristiano Girardi Editore, Bologna

Lucisano, A., Severino, L., Crescenzo, G., *et al*., 1993. Avvelenamenti da pesticidi negli animali da compagnia nell'isola di Ischia. *Acta Medica Veterinaria*, **39**, 203–212

Meucci, V., Giorgi, M., Loni, R., Soldani, G. and Mengozzi, G., 2002. Intossicazione in medicina veterinaria: casistiche del laboratorio di tossicologia della facoltà di medicina veterinaria di Pisa nel triennio 1999–2001. *Atti Società Italiana di Scienze Veterinarie*, LVI, 235–236

Nebbia, C., Ghiaccherino, A,R. and Scotti, C., 1999. Tossicosi esogene dei carnivori domestici. *Il Progresso Veterinario*, **4**, 173–177

Veterinary Research Communications, 28 (2004) 97–102

Regional Regulations Prohibiting the Use and Possession of Poisoned Lures

G. Brajon
Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana
Correspondence: Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Via Castelpulci 41, 50010 San Martino alla Palma (FI,) Italy
E-mail: gbrajon@rm.izs.it

Keywords: lures, poisoned food scraps, prohibition of use, laws, penalties

The recent decree of the President of the Council of Ministers of 28 February 2003, which acknowledges the agreement between the Ministry of Health, the Regions, and the Autonomous Provinces of Trento and Bolzano on the subject of the well-being of pets and pet therapy, provides that the Regions adopt – within the framework of their own competencies – provisions aimed at:

1. guaranteeing the well-being of pets
2. avoiding both direct and indirect reprehensible uses
3. the institution of a system of identification by means of subcutaneous microchips
4. utilising pet therapy with the elderly and children in need

As far as the well-being of pets is concerned, the problems linked to poisoning are one of the aspects which has most greatly involved public opinion, and in the past has led the Regions to introduce new legislation.

Article 21 of Law No. 157 of 11 February 1992 established "regulations for the protection of warm-blooded wild fauna and for game sampling", prohibiting the use of poisoned lures or food scraps and the application of penalties: namely, a fine of up to € 1,549.37.

Furthermore, depending on the particular case, the legislation allows for further hypotheses of a criminal offence as provided for by the Penal Code.

The sale of poisonous substances, utilised above all in the extermination of rats, is regulated by Royal Decree No. 1965 of 27 July 1934, which obliges vendors to keep a register in which are noted the personal particulars of the purchaser, the job or profession exercised, the substance purchased, and the relative quantity.

Recently, in addition to sales, it has become necessary to extend the regulations to include the preparation, possession, and distribution of poisoned lures or food scraps.

Until now, only two Regions – namely, Tuscany and Umbria – have promulgated laws dealing with the prohibition of the possession and use of poisoned lures, integrated in the 1992 Law (see References). The aims of the laws are to safeguard animals and human health, to ensure public hygiene, and to protect the environment.

Article 1 ratifies the prohibition to possess and/or prepare any sort of food containing toxic substances with the exception of that required for the extermination of rats.

Article 2 describes the procedures for the extermination of rats, already described in regulations in force at present but are better defined as regards to areas and information. In the Region of Tuscany, an explicit consent on the part of the owner or of those having the right, must exist before instituting procedures to rid premises, buildings, habitations, deposits, offices or private work-yards of rats. In the Region of Umbria, rat extermination is only permitted in private places subject to a communication being made by the owner (or others having the right) to the Commune and to the Local Health Unit at least 15 days before the event.

Any other non-private and non-delimited area to be submitted to rat extermination must be authorised by the Commune, indicating the substances to be used and the duration of the treatment. Furthermore, the areas must be indicated by means of special Tables which detail the intervention and the person in charge of the treatment. A system of Registers of the treatments carried out in the territory of jurisdiction has also been instituted.

Article 3 describes the administrative sanctions for those who fail to comply with the first two articles. In the Region of Tuscany, these sanctions amount to € 1,549.37, while they fluctuate between € 103.29 and € 619.74 in the Region of Umbria. To the fines is added the precautionary seizure of the poisoned lures or food scraps. In addition, if the person violating the regulation holds licences or permits in spheres of faunal and/or agro-silvo-pastoral activities or the collection of spontaneous products of the woods, suspension of the licences or authorisations for one year is provided for. In the case of repeated violations, such licences will be revoked permanently.

If the lures are scattered by special security guards or voluntary guards, in view of the seriousness of the action and the institutional role that these hold, the fine is doubled and the appointment is definitively revoked.

Administrative penalties are applied by the Province, which also transmits the documents for the possible revocation of the licences, authorisations, or appointments to the interested organisations for subsequent execution.

Interventions of surveillance, prevention, and control of the territory are provided for in Article 5, by means of a decontamination of the areas affected by episodes of poisoning under the care of the Communes in collaboration with the local Health Units and the Provincial Police. These activities may avail themselves of the aid of the Voluntary Environmental Guards (in Tuscany) or Ecological Guards (in Umbria), as well as of the owners/managers of the properties by means of a co-ordination between the Provincial and Municipal Police forces.

If the episodes continue in time, at the request of the Communes, under the care of the Provinces, delimitation of the perimetric area and the points of access is provided for, with notices to warn of the danger of poisoning without interrupting the faunal and/or agro-silvo-pastoral activities and collection of the spontaneous products of the woods.

The important role of veterinarians is described in Article 6. If veterinary surgeons

come to learn of a case of poisoning during the exercise of their private practice or of surveillance in public health, they must report the episode to the Provincial Police or to the Commune within 24 h, using a special form, and – for the Region of Umbria – also to the Local Health Unit competent for the territory.

For the Region of Tuscany, the report form is attached to the law in question and distributed to veterinarians by the Provinces, while preparation of the form for the Region of Umbria was provided for in other legislative regulations.

The veterinarian must also send samples of the organs, tissues or excrement to a regional laboratory for analysis, as well as the poisoned lures or food scraps discovered, for further diagnostic tests capable of identifying the poisonous substance.

Should the aforesaid procedures not be carried out, the Communes for the Region of Umbria and the Provinces for the Region of Tuscany may apply a pecuniary penalty of from € 25.82 to € 103.29 which, in the case of repetition, may be transformed into a report to the competent Order of Veterinary Doctors, for possible disciplinary measures.

The obligatory nature of the laboratory tests has made it necessary for the Regional Councils to identify, within the framework of the regional health service, at least one reference laboratory which is able to investigate a primary list of 14 poisons, as reported in Article 7 (Table I). The choice fell on the Experimental Institutes of Zooprophylaxis, so called *Istituti Zooprofilattici Sperimentali (IZS)*, competent for the territory.

The Region of Umbria has established that samples can be transferred to the laboratory directly by the veterinarian or through the local Health Unit. It has also arranged for special forms to be used for sending and reporting them to the Provinces, Communes and Local Health Units, as well as Tables to be utilised in rat extermination activities and the delimitation of the territories during decontamination.

The laws also provide for response times relative to the laboratory analyses and the procedures for sending test reports, which the Region of Tuscany has inserted in a

TABLE I
Basic list of the 14 poisons to be investigated in the samples sent to the reference laboratories provided for by the regional laws

Poisons	
1. Strychnine	8. Chlorates
2. Crymidine	9. Anticoagulants
3. Zinc phosphide	10. Paraquat
4. Cyanides	11. Arsenic
5. Organophosphates – carbamates	12. DNOC
6. Triazenic herbicides	13. Chloralose
7. Metaldehyde	14. Imidacloprid

special article, while the Region of Umbria makes reference to a subsequent act of the Regional Council.

Thus, for the Region of Tuscany, the terms for the analyses are 10 days from the arrival of the sample, but are flexible depending on the type of investigation to be carried out. The Region of Umbria states the times with greater precision: these fluctuate from 7 to 15 days, in relation to the typology of the substance to be investigated.

The test reports are transmitted via fax or e-mail to the veterinarian who sent the sample, to the Commune, to the Provincial Police, and – for the Region of Umbria – also to the Local Health Unit.

As far as coverage of the costs for shipping and analyses is concerned, the Region of Tuscany has inserted the analysis laboratory at the IZS of Florence into the Regional Health Plan in force. For the moment, it has not determined the cost of the toxicological analyses. For collection of the samples, in consideration of the complex nature of the territory, a network has been set up that avails itself of the organisation of police, environmental and animal-rights support associations, veterinary clinics and surgeries. The samples collected are transferred by the districts of the Veterinary Services of the Local Health Units to the Sections of the IZS. From here, the Institute completes the transport with its own vehicles to the chemical laboratory of the Florence Section.

For the cost of the analyses, the Region of Umbria refers to the price list for services of the IZS, while shipment of the carcasses or samples is made free of charge through the competent veterinary services of the Local Health Units. Upon request, also those coming from veterinary doctors in private practice.

The subsequent two Articles deal with two related aspects, which are aimed above all at prevention by means of the preparation and publication, under the care of the Provinces, by January 31st of every year, of an epidemiological map of the episodes of poisoning relative to the preceding year, and the establishing of a list of poisonous substances which must only be on sale under a regimen controlled by means of special registers. This list must be updated every two years and published in the official bulletins of the respective Regions. Through cartography, monitoring can be carried out for a better knowledge of the distribution of episodes in the territory. Furthermore, if there are several substances widely used in the preparation of poisoned food stuffs, these must be sold under strict supervision, in order to ensure their correct use.

The Region of Tuscany provides for the setting up of a special technical consultative board of policy and surveillance for the control of the law; while the Region of Umbria avails itself of the pre-existing Regional Committee for the Protection of Animals. The number of participants on the boards varies from region to region, but has several elements in common.

The Region of Tuscany also establishes the minimum three-monthly frequency for the Board's convocation.

The final Article regards the financial regulations. In 2001 the Region of Tuscany

provided financing of € 15,493.71, to be divided between the Provinces in relation to the agro-silvo-pastoral area; however, in successive years, this will have to be transformed into self-financing on the part of the Provinces, with the collection of the revenues deriving from fines. Instead, the Region of Umbria has provided for the allocation under the regional chapter "Prophylaxis, veterinary restoration to health", and the IZS, to cover the costs of shipment and analysis of the samples, providing for an annual determination of the amount of the costs with the Regional financial law. Also provided for were resources for covering the costs of the Regional Committee for the Protection of Animals.

Now at a distance of two years from the issuing of the laws, a first review must be made. In Tuscany, application of the regulation enabled a comparative evaluation to be carried out, during the first six months of 2002, through meetings realised by the Order of Veterinary Doctors at a provincial and inter-provincial level between veterinarians, provincial administrations, the state Corps of Forestry Service rangers, and associations of volunteers.

The meetings revealed a sensitivity to the problem and interest in collaborating for application of the law; nevertheless, several problems linked to a uniform application of the regulations in the territory emerged.

A fundamental aspect is that of verifying the appropriateness of the demand and, thus, the subsequent availability of reliable epidemiological data through the necessary filter of the veterinary doctor. It was sought to streamline the route for reporting and sending the material to the laboratories, by means of the use of a single report form and the sending of samples through Internet. To this end, the Region of Tuscany has activated a special space on its own web site. One problem seems to be the requirement to make a report for uncertain cases or for incidents in which intention to act with malice is not recognised.

The possibility that the owner refuses to have an autopsy performed can represent an obstacle to the sending of pathological material to be submitted to toxicological investigations. Consequently, the Region of Tuscany has initiated an information campaign to sensitise the public regarding the application of the law. In several cases, difficulties have been registered regarding carrying out the autopsy in the surgeries; moreover, transportation of the samples must be effected according to the recent MINSAL (Ministry of Health) circular No. 5 of 8/05/2003, "Recommendations for safety in transporting infectious materials and diagnostic samples".

The role of the Veterinary Services of the Local Health Units, to which the Region of Umbria has recognised specific functions, must be better clarified in Tuscany, especially in the link with the Communes for the flow of information and epidemiological surveillance. Also, no uniform criteria have been adopted on the part of the Provinces for preparing the annual cartography, with the consequent difficulty in collecting data at a regional level.

As far as the analytical laboratory activity and training are concerned, availing itself of a protocol of agreement stipulated with the Region of Tuscany, an integration

has been initiated between the IZS and the Faculty of Veterinary Medicine of Pisa, in order to make the services rendered more efficient and efficacious.

In Tuscany, the link-up with the Bill on the protection of animal rights through the setting up of the regional National Veterinary Health Service can be interesting: in addition to identifying the recipients of the service and the criteria and procedures for the conventions between private structures and public administrations, it must also define the minimum levels of the services.

REFERENCES

Legge 11 febbraio 1992, n. 157. Norme per la protezione della fauna selvatica omeoterma e per il prelievo venatorio,*Gazzetta Ufficiale* 25 febbraio 1992, n. 46, S.O.

Legge Regione Toscana 16 agosto 2001, n. 39. Norme sul divieto di utilizzo e detenzione di esche avvelenate, *Bollettino Ufficiale della Regione Toscana* n. 27 del 27 agosto 2001, Parte I, Sezione I

Legge Regione Umbria 22 ottobre 2001, n. 27. Norme in materia di divieto di detenzione ed utilizzazione di esche avvelenate, *Bollettino Ufficiale della Regione dell'Umbria* n. 53 del 26 ottobre 2001, Parti I, II (serie generale)

Regione Umbria – Deliberazione della Giunta Regionale del 27 marzo 2003, n. 362. L.R.N. 27/01 concernente norme in materia di divieto di detenzione ed utilizzazione di esche avvelenate. Linee di indirizzo e coordinamento vincolanti.

Veterinary Research Communications, 28 (2004) 103–106

Laboratory Diagnostic Examinations in Veterinary Toxicology

M. Giorgi[1] and B. Naso[2]*
[1] *Dipartimento di Clinica Veterinaria, Università degli Studi di Pisa, V.le delle Piagge 2 – 56124 Pisa;* [2] *Dipartimento di Patologia e Sanità Animale, Università degli Studi di Napoli Federico II, Via Veterinaria 1 – 80137 Napoli*
* *Correspondence: Dipartimento di Patologia e Sanità Animale, Facoltà di Medicina Veterinaria, via Veterinaria 1, 80137 Napoli, Italy*
E-mail: barbaranaso@yahoo.com

Keywords: baits, clinical-toxicological schedule, laboratory analysis, sample collection

INTRODUCTION

One of the main activities in a laboratory of veterinary toxicology is to detect, in organic and inorganic matrices, substances suspected to be responsible for individual and collective poisonings of domestic and wild animals (Giorgi *et al*., 2002). The veterinary surgeon who asks for a toxicological analysis should keep to some elementary rules, which allow quick and cheap identification of the toxic substance. There are two main steps: 1) the proper collection, packaging and delivery of samples; 2) the compilation of a detailed and accurate clinical-toxicological schedule. Unfortunately, our experience has shown that the above-mentioned rules are almost always unknown. Consequently, samples are frequently sent to the laboratory in unsuitable conditions, which make toxicological analysis impossibile.

The experiences of the laboratories of Veterinary Toxicology of the Universities of Naples and Pisa are discussed in this communication. In particular, correct ways for collecting, packaging and delivering samples and an example of a clinical-toxicological schedule are treated. Some of the baits recently recovered in the Tuscany region are also illustrated.

COLLECTION, PACKAGING AND DELIVERY OF SAMPLES

Organic matrices to be collected are different, depending on which toxic substance is responsible for poisoning. If the nature of the chemical is known, it is possibile to consult data about its toxicokinetic properties and to collect the most suitable samples. In the absence of this knowledge, specimens such as vomitus, blood, urine, liver, stomach or rumen contents, kidney and fat may be particularly useful for toxicological analyses. Samples should be submitted in suitable quantities: 5 ml of serum; 10 ml of whole blood; 50 ml of urine; 200 g of bait, vomitus, gastric or rumen content; 100 g each of liver, kidney and fat. The entire brain should be submitted.

With the exception of only very small species such as canaries, small rodents etc, the whole carcass does not need to be delivered. When the bait is found, it must always be sent to the laboratory.

Each specimen should be put into a single plastic container (if possible, suitable for contact with food) that can be tightly sealed. One must avoid:

- the use of plastic bags or glass containers, which break easily with consequent loss of sample;
- the addition of antiseptics, preservatives and fixatives, which may interfere with the chemical analysis;
- contact of the sample with adsorbent substances, such as cotton-wool, gauze, cloth or paper, which may cause its desiccation.

Indications (owner's name, animal name or number, and type of specimen) must be reported on the container on a label or written directly on it by means of an indelible pen.

In order to avoid the degradation of chemicals, tissue specimens should be quickly frozen, whereas serum and blood should be refrigerated. Samples must be delivered by an express carrier, in an insulating container, such as a polystyrene box, where they should be surrounded with adsorbent materials such as sawdust or newspaper. In any case, samples must be kept cool during the delivery. A clinical-toxicological schedule, not in direct contact with the samples, must be sent to the laboratory together with them (Lorgue *et al.*, 1999; Peterson and Talcott, 2001).

CLINICAL-TOXICOLOGICAL SCHEDULE

The compilation of the clinical-toxicological schedule is very important, in order to allow the laboratory to carry out the analysis in a proper way. This should contain all information, which may address the toxicologist to intelligently select which toxicant to analyse for, especially when the toxic identity is unknown. In fact, analytical methods for the research of xenobiotics in organic and inorganic matrices are highly specific and they aim at the detection of only one chemical or, at least, of a group of substances having a very similar chemical structure. In addition, the correct compilation of the clinical-toxicological schedule by the veterinary surgeon allows responsible bodies to gather information about modalities, types and places of poisonings in order to create an updated database of intoxications in domestic animals. Figure 1 shows the clinical-toxicological schedule usually given by the Veterinary Toxicology Section of Naples to veterinary surgeons who attend the Specialization Schools of the Faculty of Veterinary Medicine in Naples.

POISONING BAITS

As previously mentioned, when the bait suspected to be responsible for intoxication is found, it should always be sent to the laboratory of veterinary toxicology: it could

CLINICAL-TOXICOLOGICAL SCHEDULE

Poisoning date:

Poisoning outcome:

Time spent from the onset of the symptoms and the aid of the veterinary surgeon:

Full name, address and telephone number of the veterinary surgeon:
Full name, address and telephone number of the owner:

Signalment

Species: Breed: Age: Gender: Weight:

Anamnesis

Place in which the animal lives (city or farm; home, courtyard, garden):

Where does it usually eat and drink?

Date, hour and composition of last meal:

Does it usually eat, lick, gnaw anything?

Does the occupation or hobby of the owner predispose to the presence of any particular poisons in the home?

Any previous diseases

Any previous poisonings:

Treatments for parasites or other medications (proximate principle, dose, n° of exhibitions, date of beginning and ending of the treatment) within the past two or three weeks:

Any recent environmental treatments with potential toxic chemicals (insecticides, rodenticides, fungicides, herbicides, etc.):

Did other animals recently exhibit a similar symptomatology?

Did you know of other poisonings in the same area?

Observed clinical signs:

Medications given and their response:

Post mortem findings:

Suspect diagnosis:

Material sent for the analysis:

Bait

Did you find any bait?

What type of bait have you found?

Figure 1. The clinical-toxicological schedule provided by the Veterinary Toxicology Section of Naples veterinary surgeons

be essential for resolution of the case. All the poisoning baits submitted to the laboratory of Veterinary Toxicology of Pisa have been used with malicious intention. Some of these caused a great concern both to animal and human health because they were found in public and private areas (i.e. gardens). Unfortunately, this practice has become widespread in the Tuscany region, so much so that the police have instituted a special team to fight against the producers of poisoning baits.

A lot of poisoning baits are sent to our laboratory every year, thus we have appreciated a trend of development both in their preparation and toxicological power.

Classical baits were simply prepared, using discard organic matrix blended with very dangerous toxic substances (strychnine, cholinesterase inhibitors, metaldehyde etc.). More recently, time-consuming baits have been found such as emptied chicken necks, filled up with a toxin substance and tied up with a string, or hollowed out cheese, filled up with a toxic and covered with a lid of cheese. Successively, we received baits that contained two different toxic substances. This expedient may have been carried out in order to hide pathognomonic symptoms due to a single toxic substance and, consequently, to confuse the veterinarian in detoxification therapy practice. The most evolved bait was found a few months ago: an original *in vivo* bait (Meucci *et al*., 2003). A clay-like matrix (analysed to be a mixture of pirimicarb and endosulfan) was inserted in the subcutaneous tissue of the neck region of a female pheasant and the animal was tied to a tree. The skin close to the subcutaneous implant contained several stitches. It is important to note that whoever prepared the above-mentioned bait is able to use anaesthetic drugs (if they have been used) and to apply stitches. In conclusion, we can note that more toxic and novel baits are tending to replace classical baits.

REFERENCES

Giorgi, M., Meucci, V., Loni, R., Soldani, G. and Mengozzi, G., 2002. Intossicazioni in medicina veterinaria. *Obiettivi & Documenti Veterinari*, **6**, 57–62

Lorgue, G., Lechenet, J. and Rivière, A., 1999. *Tossicologia Clinica Veterinaria,* Cristiano Girardi Editore, Bologna

Meucci, V., Giorgi, M. and Mengozzi, G., 2003. Tossicologia veterinaria: descrizione di un caso in un fagiano. *Obiettivi & Documenti Veterinari*, **7**, 55–56

Peterson, M.E. and Talcott, P.A., 2001. *Small Animal Toxicology* (W.B. Saunders Company, Philadelphia)

Veterinary Research Communications, 28 (2004) 107–110

Follicular Waves in Cattle

J.F. Roche
Department of Animal Husbandry and Production, Faculty of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland
Correspondence: Department of Animal Husbandry and Production, Faculty of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland
E-mail:james.roche@ucd.ie

The follicle plays a fundamentally important role in reproduction and hence understanding the mechanisms of its growth, development and ovulation are important to improve and control reproductive function in farm and companion animals. Follicles become responsive to FSH at the antral stage through the development of FSH receptors on granulosa cells. FSH causes an increase in granulosa cell numbers, oestradiol and inhibin synthesis, insulin-like growth factor (IGF) production and growth of the follicle. The secretion of FSH is constitutive, resulting in its secretion into the blood stream following synthesis. GNRH plays a role in FSH secretion because immunization of heifers against it suppresses its secretion (Crowe, 1999). The balance of supply of inhibins and activin within the anterior pituitary gland is important in determining FSH output as well as the concentration of oestradiol, which has a negative feedback on FSH.

PATTERNS OF FSH IN CATTLE AND FOLLICLE WAVES

There are recurrent transient increases in FSH during the oestrous cycle, in postpartum anoestrus, prior to puberty and up to the last 1–2 months of pregnancy. However, the patterns of LH and FSH are divergent, pointing to the fact that GNRH plays an important role in determining FSH concentrations. Following the LH/FSH pre-ovulatory surge in cattle, oestradiol concentrations decline and progesterone concentrations in follicular fluid increase as granulosa cells develop LH receptors and begin the transition to luteal cells. This peri-ovulatory decrease in oestradiol and inhibins results in an increase in FSH concentrations (Adams *et al*., 1992; Sunderland *et al*., 1994) and the emergence of the first follicle wave on days 1 to 3 of the oestrous cycle. The follicles that emerge and grow to 4–6 mm are all oestrogen active follicles and through their secretion of oestradiol and inhibins, FSH concentrations begin to decline and reach basal concentrations by day 4 to 5 of the cycle.

FSH is the key hormone that regulates the regular occurrence of two or three follicle waves during the oestrous cycle of heifers. Each wave is about 7 to 10 days in duration. The first 3 days of a wave are characterized by a transient rise in basal serum FSH concentrations, a selection phase that results in a reduction in the number of growing follicles in the initial cohort from 15–25 3-mm follicles to 6–7 follicles

≥ 5 mm in diameter, and a decline in serum FSH concentrations coincident with onset of atresia of all but a single ~ 8-mm dominant follicle. The remainder of a nonovulatory wave (postselection) is characterized by growth and differentiation of a single dominant follicle to potential ovulatory size during a period of relatively low serum FSH concentrations. Specifically, the dominant follicle has an enhanced capacity to produce oestradiol as it reaches 8–9 mm in diameter, an increased amount of FSH receptors and/or its mRNA in granulosa cells by 11–12 mm in diameter. Loss of dominance marks the end of a wave and is characterized by decreased production of oestradiol, atresia or ovulation of the dominant follicle depending on stage of the oestrous cycle the wave develops, and emergence of a new follicular wave.

Current evidence indicates that follicle cohort members all grow at similar rates following emergence up to 6 mm or so, but there may be a hierarchy in size of the follicles with the largest member more likely to become the future dominant follicle (Ginther *et al*., 2001). There is a distinct change in growth rate of the largest two follicles (F1 and F2) at around 7 mm with the F1 becoming the dominant follicle while the F2 eventually succumbs to atresia. Although the end of selection and attainment of dominance, where growth deviation occurs, can be detected by ultrasound scanning of ovaries in cattle (Mihm *et al*., 1997), the biochemical signals that cause only one follicle to continue growth and development are now being elucidated.

Differential responsiveness of follicles to FSH may be regulated by intrafollicular factors such as oestradiol, progesterone, inhibins activin-A, follistatin and insulin-like growth factor binding proteins (IGFBP). These factors are synthesized locally in follicles, are regulated at least partially by FSH and may have important autocrine or paracrine roles in modulation of FSH action and hence, follicular growth and function. Potential intrafollicular factors involved in follicle selection were identified by aspirating follicular fluid from multiple individual follicles from the same heifer. The future dominant follicle was associated with highest oestradiol content in follicular fluid and the absence of detectable levels of IGFBP-4 (Mihm *et al*., 2000). FSH plays a key role in inducing the aromatase enzyme system in bovine granulosa cells. This suggests that the future dominant follicle is more sensitive to FSH due perhaps to increased FSH receptors or enhanced cell signal transduction mechanisms required for continued oestrogen synthesis. In addition, the lower IGF-B4 4 levels should result in increased bioavailability of IGFs within the follicular fluid, which should lead to proliferation of and increased FSH receptors on granulosa cells, increased oestradiol synthesis and maintain the health of the follicle. Rhodes *et al*. (2001) showed that selection of the dominant follicle was related to increased aromatase activity, increased cycle AMP in response to LH, increased oestradiol synthesis and decreased concentrations of IGFBP-4 and 5 within two days of ovulation. Thus, the bioavailability of IGFs is a key determinant of follicle development and survival in cattle.

The regulation of IGFBPs plays an important role in follicular development which occurs either through changes in gene expression or proteolysis (Nicholas *et al*., 2002). Concentrations of IGFBP 2 and 5 seem to be regulated mainly by gene expression while changes in IGFPB 2 and 4 are affected by intrafollicular levels of

specific proteases (Monget *et al*., 2002). One of the proteases identified that degrades IGFBP-4 is the pregnancy specific protein A (PAPP-A) which is responsible for the complete degradation of intrafollicular IGFBP-4 in preovulatory follicles (Monget *et al*., 2002). In contrast, Spicer *et al*. (2001) reported that levels of IGFBP-4 and 5 were decreased by proteolysis in preovulatory follicles coincident with a reduction in levels of IGFBP-2, 4 and 5. The relative roles of gene expression and proteolysis of IGFBPs in determining the bioavailability of IGF-I within the follicle require further clarification.

In summary, our work (Austin *et al*., 2001) shows that selection of a dominant follicle from a pool of growing antral follicles is a dynamic process regulated by interactions of the gonadotrophins and intraovarian factors. Following the increase in circulating FSH concentrations, follicles of 2.5–5 mm in diameter are stimulated to grow. At this time, the five largest follicles per heifer have similar amounts of oestradiol, IGFBPs, activin-A, follistatin, and percentage of apoptotic granulosa cells. By 33 h after the peak in FSH, the two largest follicles increase in size and continue to grow while FSH concentrations decline. In addition, these follicles gain an enhanced capacity to produce oestradiol, inhibin precursors, and activin-A, but begin to lose the ability to produce follistatin while maintaining low amounts of IGFBP-2, -4, and 5 and a low percentage of apoptotic granulosa cells compared with the other follicles in the cohort. By 53 h after the peak in FSH, a dominant-like follicle can be distinguished in the cohort based on its largest size, highest oestradiol, and lowest amount of IGFBPs, though functional dominance, i.e., suppression of growth of all other cohort follicles has not yet been established. At 84 h after the peak in FSH, the selected dominant follicle is the largest follicle in the cohort, it is approximately 10 mm in diameter and contains the highest amounts of ostradiol and the lowest amount of IGFBPs and follistatin compared with the other follicles in the cohort. In contrast, the remaining follicles in the cohort (F2, F3, F4 and F5), though continuing to grow until 84 h after the peak in FSH, reach smaller maximum diameters of 5.5–8.5 mm. Thus, follicular growth is characterized by 1) increases in follicular diameter, oestradiol, and at least initially in inhibin and activin-A production; 2) a decrease in follistatin; and 3) maintenance of low amounts of low molecular weight IGFBPs and a low percentage of apoptotic granulosa cells. Follicles in the wave destined to become atretic are characterized by loss of capacity to produce oestradiol and enhanced production of low molecular weight IGFBPs. These changes not only precede atresia but also occur before the cessation of follicular growth. Based on these results the earliest intrafollicular changes that distinguish a follicle destined to become dominant from the other follicles in growing cohort are enhanced capacity to produce oestradiol and maintenance of low levels of IGFBPs.

REFERENCES

Adams, G.P., Matteri, R.L., Kastelie, J.P., Ko, J.C.H. and Ginter, O.J., 1992. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. *Journal of Reproduction and Fertility*, **94**, 177–188

Austin, E.J., Mihm, M., Evans, A.C.O., Knight, P.G., Ireland, J.L.H., Ireland, J.J. and Roche, J.F., 2001. Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. *Biology of Reproduction*, **64**, 839–848

Crowe, M.A., 1999. Gonadotrophic control of terminal follicular growth in cattle. *Reproduction of Domestic Animals*, **34**, 157–166

Ginther, O.J., Beg, M.A., Bergfelt, D.R., Donadeu, F.X. and Kot, K., 2001. Follicle selection in monovular species. *Biology of Reproduction*, **65**, 638–647

Mihm, M., Good, T.E.M., Ireland, J.L.H., Ireland, J.J., Knight, P.G. and Roche, J.F., 1997. Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers. *Biology of Reproduction*, **57**, 1328–1337

Mihm, M., Austin, E.J., Good, T.E.M., Ireland, J.L.H., Knight, P.G., Roche, J.F. and Ireland, J.J., 2000. Identification of potential intrafollicular factors involved in selection of dominant follicles in heifers. *Biology of Reproduction*, **63**, 811–819

Monget, P., Fabre, S., Philippe, M., Lecerf, F., Elsen, J-M., Mazerbourg, S., Pisselet, C. and Monniaux, D., 2002. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domestic Animal Endocrinology*, **23**, 139–154

Nicholas, B., Scougall, R.K., Armstrong, D.G. and Webb, R., 2002. Changes in insulin- like growth factor binding protein (IGFBP) isoforms during bovine follicular development. *Reproduction*, **124**, 439–446

Rhodes, F.M., Peterson, A.J. and Jolly, P.D., 2001. Gonadotrophin responsiveness, aromatase activity and insulin-like growth factor binding protein content of bovine ovarian follicles during the first follicular wave. *Reproduction*, **122**, 561–569

Spicer, L.J., Chamberlain, C.S. and Morgan, G.L., 2001. Proteolysis of insulin-like growth factor binding protein during preovulatory follicular development in cattle. *Domestic Animal Endocrinology*, **21**, 1–15

Sunderland, S.J., Crowe, M.A., Boland, M.P., Roche, J.F. and Ireland, J.J., 1994. Selection, dominance and atresia of follicles during the oestrous cycle of heifers. *Journal of Reproduction and Fertility*, **101**, 547–555

Veterinary Research Communications, 28 (2004) 111–119

Manipulation of Ovarian Function for the Reproductive Management of Dairy Cows

W.W. Thatcher[1]*, J.A. Bartolome[1], A. Sozzi[1], F. Silvestre[1] and J.E.P. Santos[2]
[1] *Department of Animal Sciences, IFAS, University of Florida, Gainesville, FL, 32611-0920 and Veterinary Medicine Teaching and Research Center;*
[2] *University of California-Davis, Tulare, CA 93274, USA*
* *Correspondence: Department of Animal Sciences, IFAS, University of Florida, Gainesville, FL, 32611–0920*
E-mail: thatcher@animal.ufl.edu

INTRODUCTION

Herd reproductive efficiency is a major component leading to the economic success of the commercial dairy production unit. The modern day lactating dairy cow is considered to be sub-fertile in which the physiological stresses of high production and current management systems have a negative impact on both estrus detection and fertility. The high levels of milk production, dry matter intake, nutritional status, and metabolism of the cow appear to have altered both ovarian follicle development and luteal status. These alterations have contributed to low rates of estrus detection, increased multiple ovulations, and reduced embryo survival. With our current understanding of the factors controlling ovarian follicular dynamics and CL function, systems to manipulate the ovary for timed insemination, re-synchronization of timed inseminations for cows that do not conceive, and strategies to improve embryo survival have been developed to improve reproductive performance.

Pharmacological control of the estrous cycle involves synchronization of follicular development that is coupled with the timely induction of corpus luteum (CL) regression, and synchronization of ovulation to improve pregnancy rate. Veterinarians and producers need to understand how these systems work in order to obtain compliance and optimal results. In lactating dairy cows the tools available for reproductive management are somewhat limited due to regulated use of such hormones as estradiol, GnRH, progestins, gonadotrophins and $PGF_{2\alpha}$ that varies among countries. Any use of a hormone should be in a manner that mimics normal physiological controls and responses of the reproductive system. Pregnancy rate (PR) of the herd is the proportion of pregnant cows relative to all eligible cows to become pregnant at every estrous cycle past the voluntary waiting period. Thus pregnancy rate is a function of estrus detection rate and conception rate. Ferguson and Galligan (1993) reported that PR at the first postpartum AI explained 79% of the variation in calving interval. It is also clear that after first insemination strategies need to be developed to identify and re-synchronize the non-pregnant cow in an efficient manner to achieve a

good herd pregnancy rate. Development of timed insemination programs with normal fertility has the ability to optimize service rate and thus improve PR in herds with low estrus detection rates. The challenge is to manipulate ovarian and embryo development in a manner to have an impact on conception rate and pregnancy losses. These two components that influence herd pregnancy rate are much more difficult to impact.

ESTRUS SYNCHRONIZATION PROTOCOLS

Prostaglandins

Since the mid 1970s, $PGF_{2\alpha}$ has been the foundation system used in most dairy herds to synchronize estrus. Single or multiple injections of $PGF_{2\alpha}$ or its analogues causes a responsive CL to regress and cows return to estrus in 2 to 7 days. However, presence of anestrous cows and low rates of estrus detection will have a major impact on the number of cows responding to a $PGF_{2\alpha}$ program with detected estruses. Corpora lutea that are less than 5 days of the estrous cycle are non-responsive to $PGF_{2\alpha}$. Therefore, a single injection of $PGF_{2\alpha}$ given at random should induce estrus in approximately 70% of the cows. Approximately, 90% of the cows will have an induced estrus response when two injections of $PGF_{2\alpha}$ are given 10 to 14 days apart. Our experience is that approximately 25% of lactating dairy cows approaching a voluntary waiting period of approximately 60 days are anovulatory and of course would be non-responsive to a $PGF_{2\alpha}$ program. The estrous synchronization program with $PGF_{2\alpha}$ does not lend itself to a singled timed insemination because there is no control over follicle development. This contributes to the variability in detected estruses between 2 to 7 days after $PGF_{2\alpha}$ injection.

GnRH and Prostaglandins (Selectsynch)

Injection of GnRH at random stages of the estrous cycle followed 7 days later by an injection of $PGF_{2\alpha}$ increased the number of dairy heifers synchronized within a 5-day period and enhanced the precision of synchrony during days 2 and 3 after injection of $PGF_{2\alpha}$ (Thatcher *et al*., 1989). Treatment with GnRH results in a LH surge and ovulation of a dominant follicle ($\geq$10 mm) leading to recruitment of a new cohort of follicles. GnRH induced ovulation is critical for recruitment of a new follicle wave, and the highest response to an GnRH injection is observed when it is given between days 5 and 9 of the cycle when there is a first wave dominant follicle that is responsive to LH (Vasconcelos *et al*., 1999). The system of giving a GnRH injection followed 7 days later by $PGF_{2\alpha}$ (Selectsynch) results in acceptable estrus (67.2%), conception (41.5%) and pregnancy (30.2%) rates with lactating dairy cows (Burke *et al*., 1996; Santos *et al*., 2001).

OVULATION SYNCHRONIZATION PROTOCOLS

With the ability to synchronize follicular wave development coupled with $PGF_{2\alpha}$ induced regression of the CL, it was possible to implement a precise synchronization of ovulation for a Timed Insemination with an acceptable conception rate to first service.

Ovsynch

The Ovsynch protocol was developed as a breeding strategy to eliminate the need for estrus detection. The protocol is composed of an injection of GnRH at random stages of the estrous cycle to induce ovulation of the dominant follicle and synchronize a new follicle wave emergence. Seven days later, $PGF_{2\alpha}$ is given to regress the original and the newly formed CL, followed by a second GnRH injection 48 h later to induce a synchronous ovulation 24 to 32 h later. A timed insemination is carried out at 12 to 16 h after the second GnRH injection (Fig. 1). This protocol has been implemented very successfully in many commercial dairy farms world wide as a strategy for AI during the first postpartum service, as well as for re-insemination of non-pregnant cows. Although the Ovsynch protocol allows for TAI without the need for estrus detection, approximately 10 to 15% of the cows will display signs of estrus during the protocol and they should be inseminated promptly if maximum PR is to be achieved.

Lactating dairy cows from three commercial herds ($n = 333$) were randomly assigned to either the Ovsynch protocol or AI based on estrus detection with periodic use of $PGF_{2\alpha}$ (Pursley *et al.*, 1997). Pregnancy was diagnosed 32 to 38 d after AI, and non-pregnant cows were re-inseminated using the original treatment. Median days postpartum to first AI (54 vs 83; $p < 0.001$) and days open (99 vs 118: $p < 0.001$) were lower for cows in Ovsynch compared to cows inseminated following estrus detection. Therefore, implementation of TAI with the Ovsynch protocol for the first postpartum AI improved reproductive performance in dairy cows, and subsequent re-insemination of open cows with TAI resulted in higher PR. When measuring PR,

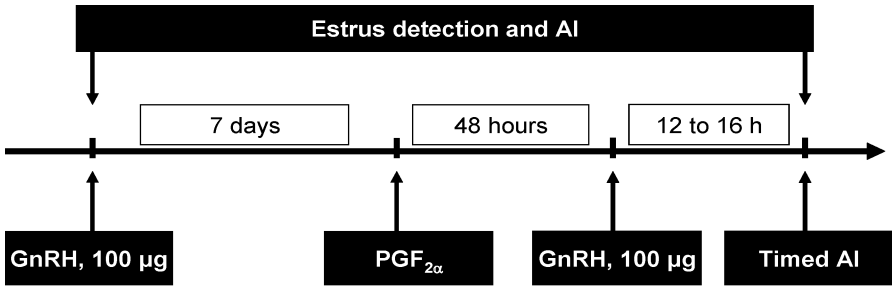


Figure 1. Ovsynch protocol for timed AI

Burke *et al.* (1996) demonstrated that an Ovsynch program for a first service timed insemination was as effective as inseminating lactating dairy cows at detected estrus following a synchronization program of GnRH and $PGF_{2\alpha}$ given 7 days apart.

Presynch–Ovsynch

Response to the Ovysnch protocol is optimized when cows ovulate to the first GnRH injection of the program, and when a responsive CL is present at the moment of the $PGF_{2\alpha}$ treatment. Vasconcelos *et al.* (1999) noted that initiation of the Ovsynch protocol between days 5 and 9 of the cycle resulted in the highest ovulation rate in response to GnRH. Ovulation to the first GnRH injection and initiation of a new follicular wave should improve PR because an ovulatory follicle with a reduced period of dominance is induced to ovulate (Austin *et al.*, 1999). Furthermore, initiating the Ovsynch protocol prior to day 12 of the estrous cycle should minimize the number of cows that come into estrus prior to the 2nd GnRH injection and ovulate prior to the completion of the program.

Moreira *et al.* (2001) designed a pre-synchronization protocol to optimize response to the Ovsynch program by giving two injections of $PGF_{2\alpha}$ 14 days apart, with the second injection given 12 days prior to the first GnRH of the TAI protocol (Fig. 2). The Presynch-Ovsynch program increased pregnancy rates 18 percentage units (i.e., 25% to 43%); in lactating cyclic cows. Additional strategies to pre-synchronize in order to optimize the Ovsynch program are possible but need to be well thought out in order to minimize cows being in early metestrus or late diestrus when the Ovsynch

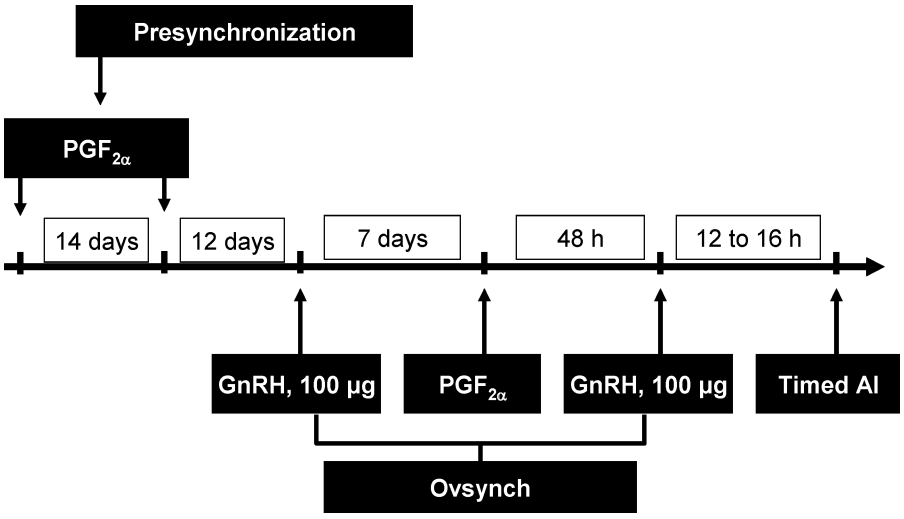


Figure 2. Presynch/Ovsynch protocol for timed AI in the first postpartum service

program is initiated. Future programs for further optimization of fertility likely will consider programs that manipulate ovarian function such that follicular turnover via ovulation or induced follicular atresia occurs in all cows, and luteal phase like progesterone concentrations are sustained until the time of induced CL regression. These future systems in lactating dairy cows may include insertion of intravaginal devices containing progesterone and acute injections of estrogens. Success of the OvSynch program is dependent on whether lactating dairy cows are anestrus or cycling. Pregnancy rates were less in cows that were not cycling at the time the OvSynch program was initiated (e.g., 22% versus 42%). Overall, the TAI protocol was able to induce cycles in 75% of anestrous cows, based upon the number of anestrous cows which were classified as ovulating to either the first and/or second injection of GnRH. If anestrous cows ovulate to the first and second GnRH injections of the OvSynch program then pregnancy rates appeared to be normal (e.g., 39%). Insertion of progestin devices as part of the Ovsynch program (i.e., between GnRH and $PGF_{2\alpha}$ injections) may also benefit anestrous animals.

Presynch–Heatsynch

An alternative strategy to control the time of ovulation is the ability of exogenous estradiol to induce a LH surge by stimulating hypothalamic secretion of GnRH when given in a low progesterone environment during late diestrus and proestrus. An estradiol induced LH surge lasts for approximately 10 h, which is comparable to a spontaneous LH surge and longer than the LH surge induced by GnRH. Estradiol cypionate (ECP), an esterified form of estradiol-17β that is available commercially for use in cattle, has been used as part of a timed insemination program in lactating dairy cows. The ECP is used to replace the second GnRH injection of an OvSynch program. Cows are pre-synchronized with two injections of $PGF_{2\alpha}$ given 14 d apart with Heatsynch beginning 14 d after the second injection of $PGF_{2\alpha}$. Cows are then injected with GnRH followed by $PGF_{2\alpha}$ 7d later. The ECP (1 mg, i.m.) is injected 24 h after $PGF_{2\alpha}$, and cows are inseminated 48 h later. Pregnancy rates did not differ between Heatsynch and OvSynch programs (Pancarci *et al.*, 2002; Fig. 3). Cows detected in estrus after ECP had a higher fertility than those not detected in estrus at the timed insemination.

In lactating dairy cows, the frequencies of detected estrus and ovulation after ECP were 75.7% and 86.5%, respectively (Pancarci *et al.*, 2002). Estrus occurred at 29.0 ± 1.8 h ($n = 28$) after ECP, lasted for 12.5 h and was associated with an average of 20.3 mounts. Mean intervals to ovulation were 55.4 ± 2.7 h after ECP and 27.5 ± 1.1 h after onset of estrus. Since 75% of the ovulations occurred between ≥ 48 h to ≤ 72 h after ECP, it is recommended that any cow detected in estrus by 1.5 days after ECP injection be inseminated at detected estrus, and all remaining cows be inseminated at 48 h. Based on synchronization of ovulation and pregnancy rates, ECP can be utilized as an alternative to induce ovulation in place of GnRH for a

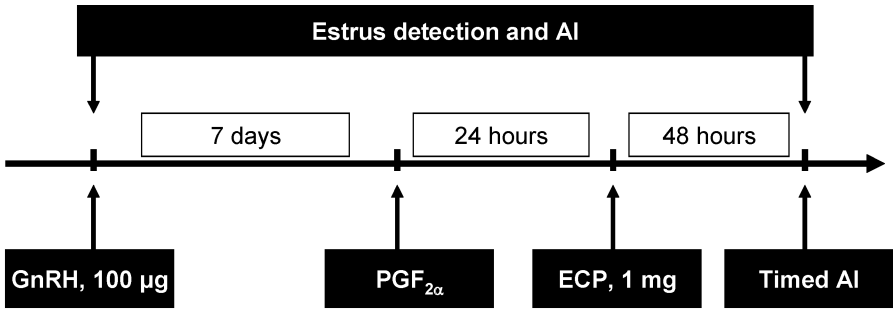


Figure 3. Heatsynch protocol for timed AI

timed insemination. Since lactating dairy cows have reduced concentrations of plasma estradiol in the preovulatory period and reduced intensity of estrus, the elevation of estradiol following ECP injection supplements for a lactational induced deficiency, and our experience indicates that cows expressing estrus are fertile. The secretion of LH is regulated directly by GnRH whereas estradiol induces LH secretion indirectly via a positive feedback stimulation of hypothalamic GnRH secretion that then stimulates LH secretion. If cows are anovulatory (e.g., anestrus or have not developed positive estradiol feedback) the Heatsynch program may not be as effective as the GnRH based OvSynch program in which GnRH causes the direct secretion of LH. Greater uterine tone, ease of insemination and occurrence of estrus with the use of the Heatsynch program are well received by inseminators. Alternatively, in facilities with concrete flooring, the reduced estrous expression associated with the OvSynch program may be preferred. Since fertility between the two programs appears to be comparable, producers have a choice, which also includes relative costs of drugs (i.e., ECP < GnRH).

Since lactating dairy cows are sub-fertile and have lower concentrations of estradiol, it is possible that increased estradiol concentrations due to ECP injection may enhance fertility. Cerri *et al.* (2003) compared fertility of cows undergoing a Presynch-Heatsynch reproductive management program vs cows inseminated at detected estrus following a Presynch-Selectsynch program. The Presynch-Heatsynch cows had both a higher conception rate (43.0 > 35.6%) and pregnancy rate (48.5 > 23.6%) at day 30 after insemination than the cows detected in heat and inseminated with the Presynch-Selectsynch program. Pregnancy losses were similar (12%) between day 30 and 58 after insemination. Apparently, increased estradiol concentration in the periovulatory period of lactating dairy cows at first service enhanced conception rate and warrants additional investigation.

RE-SYNCHRONIZATION TIMED INSEMINATION

Re-synchronization of non-pregnant cows is required if optimal herd PRs are to be achieved. Only 30 to 45% of inseminated cows are pregnant at 40 d after insemina-

tion, and the non-pregnant cows need to be re-inseminated as quickly as possible. Strategies to accomplish this with the use of timed insemination can be rather aggressive with re-synchronization of follicle development prior to pregnancy diagnosis and early pregnancy diagnosis with the use of ultrasound.

Ovsynch initiated 7 days prior to pregnancy diagnosis

A study was conducted to determine the effects of re-synchronization with GnRH on day 21 after AI on PR and losses of pregnancy in lactating dairy cows (Chebel *et al*., 2003). Holstein cows, 585, on two dairy farms were assigned to one of two treatments. On day 21 after the pre-enrolment AI, animals assigned to the re-synchronization group received an injection of 100 μg of GnRH, whereas animals in the control group received no treatment. Pregnancy was diagnosed on day 28 and reconfirmed 14 d later. Non-pregnant cows on day 28 were timed inseminated after the completion of the Ovsynch protocol 10 and 17 d after the enrolment in the study for re-synchronized and control groups, respectively. For re-synchronized and control cows, PR at days 28 (33.1 vs 33.6%; $p < 0.80$) and 42 (27.0 vs 26.8%; $p < 0.98$) after the pre-enrolment AI did not differ. Administration of GnRH on day 21 after AI had no effect on the losses of pregnancy between re-synchronized and control groups from 28 to 42 d (17.9%; $p < 0.74$) after AI. Pregnancy rate after the re-synchronization period was similar for both treatment groups and it averaged 29.4%. Therefore, re-synchronization with Ovsynch beginning on day 21 after the initial insemination and prior to pregnancy diagnosis at day 28 did not adversely affect the initial PR and pregnancy loss in lactating dairy cows, and it can potentially expedite re-insemination of non-pregnant cows. With this approach, the re-synchronization and control groups were re-inseminated at 31 and 38 days after the previous service.

Use of Ovsynch and Heatsynch beginning at 23 days after AI

In our previous studies we evaluated the interval from AI to return to estrus in cows that were nonpregnant (Fig. 4). Based on this distribution of intervals to estrus, it is feasible to inject GnRH at day 23 (i.e., 22–24 days) after insemination to synchronize the follicular wave and ensure that corpora lutea are present at day 30 that will be responsive to $PGF_{2\alpha}$. Currently, a study is underway (J. Bartolome and W.W. Thatcher, personal communication) in which GnRH is administered between days 22 and 24, and pregnancy diagnosis using US is conducted between days 29 to 32 (i.e., mean 30 days). Cows diagnosed nonpregnant receive $PGF_{2\alpha}$, and ovulation is synchronized with either ECP or GnRH (Fig. 4). The timing of the Ovsynch protocol is standard with the ovulatory dose of GnRH given 48 h after injection of $PGF_{2\alpha}$ and a TAI at approximately 16 h after GnRH. Our experiences with ECP for re-synchronization are such that ECP (1 mg) is given 24 h after injection of $PGF_{2\alpha}$, and we have

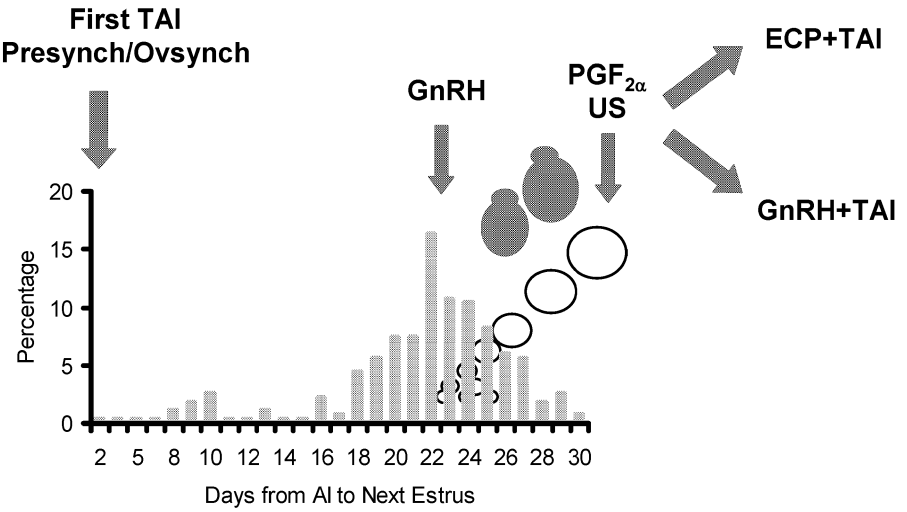


Figure 4. Future strategy for re-synchronization

chosen to time-inseminate all of the cows at approximately 36 h after injection of ECP. Results evaluating 756 cows indicate the following distribution according to stages of the estrous cycle at the time of pregnancy diagnosis: diestrus 75%, metestrus 5.8%, proestrus 9.6%, ovarian cysts 7.9% and anestrus 1.6%. In addition, 50% of the ovarian cysts showed luteinization at ultrasound. For the 567 diestrus cows, PR for re-synchronization were 28.7% (60/209) for cows subjected to PGF-ECP-TAI and 26.9% (60/223) for cows subjected to PGF-GnRH-TAI. Pregnancy losses between days 30 and 55 averaged 10% and did not differ between groups. Since we still have cows without a corpus luteum despite the fact they received a GnRH injection 7 d prior to pregnancy diagnosis, alternative protocols are being evaluated for cows in metestrus, proestrus and with an ovarian cyst.

Early detection of nonpregnant cows is crucial for enhancing reproductive performance. Cows with a CL at the time of diagnosis of nonpregnancy which were treated with GnRH 7 days earlier can be re-synchronized with $PGF_{2\alpha}$ followed by either ECP or GnRH. Cows are then TAI within 3 days of the non-pregnant ultrasound diagnosis. However, cows with CL at diagnosis of nonpregnancy which were not pre-treated with GnRH can be re-synchronized with protocols for TAI (Heatsynch and Ovsynch). In addition, ultrasound and rectal palpation allow for detection of reproductive tract abnormalities that will contribute to treatment and culling decisions. Future cow-side pregnancy tests may allow detection of nonpregnant cows at an early stage (e.g., day 23) so that re-synchronization protocols can be initiated only in cows known to be nonpregnant. Choosing the proper stage to initiate the program with GnRH (e.g., day 23) takes advantage of the re-occurring follicular wave and CL to reduce the time for re-insemination.

REFERENCES

Austin, E.J., Mihm, M., Ryan, M.P., Williams, D.H. and Roche, J.F., 1999. Effect of duration of dominance of the ovulatory follicle on onset of estrus and fertility in heifers. *Journal of Animal Science*, **77**, 2219–2226

Burke, J.M., de la Sota, R.L., Risco, C.A., Staples, C.R., Schmitt, E.J.-P. and Thatcher, W.W., 1996. Evaluation of timed insemination using a gonadotropin-releasing hormone agonist in lactating dairy cows. *Journal of Dairy Science*, **79**, 1385–1393

Cerri., R.L.A., Santos, J.E.P., Juchem, S., Galvão, K.N. and Chebel, R., 2003. Timed artificial insemination (TAI) with estradiol cypionate (ECP) or insemination at detected estrus in lactating dairy cows. *Journal of Dairy Science*, **86**(Suppl. 1), 181 (Abstr.)

Chebel, R.C., Santos, J.E.P., Juchem, S.O., Cerri, R.L.A., Galvão, K.N. and Thatcher, W.W., 2003. Effect of resynchronization with GnRH on day 21 after artificial insemination on conception rate and pregnancy loss in lactating dairy cows. *Theriogenology* (*In press*)

Ferguson, J.D. and Galligan, D.T., 1993. Reproductive programs in dairy herds. *In Proc. Centr. Vet. Conf.*, pp. 161–178

Moreira, F., Orlandi, C., Risco, C.A., Mattos, R., Lopes, F. and Thatcher, W.W., 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. *Journal of Dairy Science*, **84**, 1646–1659

Pancarci, S.M., Jordan, E.R., Risco, C.A., Schouten, M.J., Lopes, F.L., Moreira, F. and Thatcher, W.W., 2002. Use of estradiol cypionate in a pre-synchronized timed artificial insemination program for lactating dairy cows. *Journal of Dairy Science*, **85**, 122–131

Pursley, J.R., Kosorok, M.R. and Wiltbank, M.C., 1997. Reproductive management of lactating dairy cows using synchronization of ovulation. *Journal of Dairy Science*, **80**, 301–306

Santos, J.E.P., Thatcher, W.W., Pool, L. and Overton, M.W., 2001. Effect of human chorionic gonadotropin on luteal function and reproductive performance of high producing lactating Holstein dairy cows. *Journal of Animal Science*, **79**, 2881–2894

Thatcher, W.W., Macmillan, K.L., Hansen, P.J. and Drost, M., 1989. Concepts for regulation of corpus luteum function by the conceptus and ovarian follicles to improve fertility. *Theriogenology*, **31**, 149–164

Vasconcelos, J.L.M., Silcox, R.W., Rosa, G.J.M., Pursley, J.R. and Wiltbank, M.C., 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. *Theriogenology*, **15**, 1067–1078

Veterinary Research Communications, 28 (2004) 121–126

Production and Quality of Bovine Oocytes and Embryos

C. Galli*, R. Duchi, G. Crotti, P. Turini, N. Ponderato, S. Colleoni, I. Lagutina and G. Lazzari
Laboratorio di Tecnologie della Riproduzione, CIZ srl, Istituto Sperimentale Italiano Lazzaro Spallanzani, Via Porcellasco 7/F, 26100 Cremona, Italy
**Correspondence: Laboratorio di Tecnologie della Riproduzione, CIZ srl, Istituto Sperimentale Italiano Lazzaro Spallanzani, Via Porcellasco 7/F, 26100 Cremona, Italy*

ABSTRACT

Many factors influence the efficiency of the *in vitro* embryo production technology in cattle but the most important are the physiological conditions of the donor and the culture protocols for oocyte maturation and fertilization and for embryo culture from zygote to blastocyst. Therefore, general factors such as age, body conditions and herd management play a pivotal role together with more specific factors such as reproductive soundness and ovarian cyclicity. Given that good quality and competent oocytes are available a complex series of processes, including oocyte maturation, fertilization and culture of the derived zygotes, must be completed to generate viable embryos.

INTRODUCTION

In recent years modern methods of assisted reproduction have been widely applied to cattle breeding (Galli and Lazzari, 1996; Galli *et al*., 2001, 2003). From the very beginning, the IVF technique in the bovine was mainly considered the method that could allow production of large numbers of cheap embryos from the ovaries of slaughtered females for commercial purposes. While this represented an obvious and immediate application it was also an instrumental step to fundamental studies by making available large numbers of embryos for research use. Therefore, a considerable body of fundamental knowledge has accumulated and this progress has increased not only the applications but also the understanding of the basic physiological processes involved in oocyte and embryo development.

In this paper we will summarise the state of the art in assisted reproduction in bovines, including a brief analysis of the environmental, physiological and technical factors that affect the successful completion of all the steps that bring the immature oocytes to the stage of a good quality viable embryo.

DONOR ANIMALS

Any female after puberty is a potential donor of oocytes. The only requirement is the presence of antral follicles on the ovaries. Therefore, only animals with hypoplasic ovaries or in the immediate post-partum or late in pregnancy should be considered

unsuitable donors. Interestingly, certain ovarian pathological conditions such as ovarian cysts that often compromise the reproductive functions do not represent major obstacles for *in vitro* embryo production. Also pregnant animals can be good oocyte donors at least in the first months of pregnancy. General body conditions are important in determining the number of oocytes recovered and their subsequent successful development into viable embryos (see Table I). The variability of the oocyte developmental competence observed amongst donors or batches of donors is great but probably not different from that observed in conventional ET. Certainly this variability is mostly dependent on nutrition and management of the animals (Kruip *et al*., 1995) and on the correlated physiological status. The common habit of feeding cattle diets high in degradable crude protein or in excess of requirements can reduce fertility and lower uterine pH (see Butler as review, 1998; Ocon and Hansen, 2003). Recent studies have shown that high plasma levels of ammonia and urea correspond to increased concentrations in the reproductive tract and especially in the oviduct (Kenny *et al*., 2002). The altered oviductal milieu is expected to cause profound disturbances to the developing conceptus since it is known that bovine embryos exposed to different culture environments can have altered level of expression of developmentally important genes (Lazzari *et al*., 2002).

Over the years it has become clearly evident that the developmental capacity of the oocyte is also dependent on intrinsic ovarian factors. The most relevant is the follicular environment in which the oocyte has developed before collection. Primarily, the size of the follicle is very important, being correlated to the stage of oocyte development along the growth phase. The status of the follicle along the follicular wave, growing or regressing, and its consequent degree of atresia greatly affect oocyte development capacity (Galli and Moor, 1991). A recent study in which oocytes were collected from cycling donors at precise stage in the cycle, has reiterated this concept by showing that oocyte quality is in fact the major factor that determines the efficiency of embryo production (see as review Lonergan *et al*., 2001). On the other hand, more

TABLE I
Effect of general physiological conditions of donors on embryo production by IVP following recovery of the ovaries at the abattoir

	No. donors	No. oocytes (per donor)	Cleavage %	No. viable embryos (per donor)	% viable embryos/ oocytes
Healthy donors	26	1145 (44.0a)	62.8	248 (9.5a)	21.6
Terminal donors	12	281 (23.4b)	70.1	33 (2.7b)	11.7

Terminal donors were affected by chronic conditions such as lameness, chetosis, etc. Student *t*-test. Values within columns with different letters are statistically different ($p < 0.05$).

extensive studies on cycling animals with or without prestimulation with gonadotropins have shown that in general, but not always, prestimulated donors provide oocytes with higher developmental competence (see as review Hendriksen *et al*., 2000).

EMBRYO PRODUCTION FROM LIVE DONORS: OVUM PICK-UP

At the beginning of the IVP technology all oocytes were collected from slaughtered donors but in recent years the Ovum Pick Up technique (OPU) has become a routine procedure (Galli *et al*., 2001). This technological advance has considerably widened the potential application of IVP by making it possible to couple assisted reproduction and genetic improvement in cattle. It consists of the transvaginal aspiration of follicular oocytes with the aid of a scanner with an adequate endovaginal (or adapted for the vaginal use) sector probe. A guided needle secured to the probe is connected to a test tube and to a vacuum pump to aspirate the follicular fluid and the oocyte contained in it. The donor is confined in a crush, mildly sedated and given an epidural anaesthesia just before collection. OPU has virtually no drawbacks for the donor and can even have a therapeutic effect in some infertile donors affected by ovarian cystic syndrome or similar pathologies that compromise reproductive function. Virtually any female starting from 6 months of age up to the third month of pregnancy and also soon after calving (2–3 weeks), is a suitable donor (9). Young calves 2–3 months-old can also be oocyte donors although collection requires surgical laparotomy. In Table II we present data from our laboratory to illustrate the influence of the age of donors on embryo production. Unlike conventional superovulation, OPU does not interfere with the normal reproduction and production cycles of the donor. OPU can be performed sporadically or on a regular basis such as two times a week for many weeks or months. The twice a week protocol is the one that yields the maximum number of competent oocytes in a given period of time. If irregular intervals of oocytes retrieval are used or if OPU is done once a week a dominant follicle develops (Garcia and Salaheddine, 1998) and this causes the atresia of subordinate follicles making them less suitable for embryo production because of the lower quality of the oocytes. A practical tip to overcome this problem or to avoid having a donor in estrus at the time of OPU, is to administer 3–4 days prior to collection a Gn-RH analogue to pharmacologically ablate the dominant follicle and to initiate a new follicular wave (our unpublished observations). Another advantage of OPU is that it is not necessary to treat the donor with gonadotropins with the inevitable side effects. This is a very important advantage especially for young heifers in which gonadotropin-stimulation can cause mammary oedema and ovarian cystic syndrome, and for show cows where repeated superovulation can cause relaxation of the udder ligament. However, a Canadian study has demonstrated that a combination of mild superovulatory treatment with FSH and OPU can increase the number of embryos produced in a given time, therefore representing a valid alternative to conventional superovulation

Table II. Developmental capacity of oocytes collected from donors of different age with or without hormonal prestimulation

Type of donor	Donors (No.)	OPUs (No.)	Ocytes (per OPU) (No.)	Cleaved (per OPU) (No.)	Cleavage (%)	Freezable embryos (per OPU) (No.)	Transferrable embryos (per OPU) (No.)	Freezable embryos/ cleaved (%)	Transferrable embryos cleaved (%)
Calves 2–3 months of age (untreated)	8	8	128 (16.0)	64 (8.0)	50.0	4 (0.5)	6 (0.7)	4.5(a)	8.2(a)
Calves 2–3 months of age (eCG treated*)	37	37	1005 (27.2)	595 (16.1)	59.2	81 (2.2)	116 (3.1)	13.6 (b)	19.5 (b)
Calves 6–8 months of age (untreated)	15	82	795 (9.7)	485 (5.9)	61.0	61 (0.7)	85 (1.0)	12.6 (b)	17.5 (b)
Heifers from 9 months of age (untreated)	38	254	2288 (9.0)	1271 (5.0)	55.6	298 (1.2)	388 (1.5)	23.4 (c)	30.5 (c)
Cows (untreated)	59	261	2579 (9.9)	1661 (6.4)	64.4	532 (2.0)	655 (2.5)	32.0(d)	39.4 (d)

*The protocol of ovarian stimulation for 2–3 months eCG treated calves was as follows: D + 0: 5 mg estradiol valerate and intravaginal progestagen releasing sponges (80 mg fluorogestone acetate), D + 3 and D + 11: 400 IU eCG, D + 16: 1000 IU eCG, D + 18: laparotomy and oocyte collection. Student *y*-test. Values within columns with different letters are statistically different ($p < 0.05$). From Galli *et al.*, *Theriogenology*, 2001, **55**, 1341–1357.

(Bousquet *et al*., 1999). A final advantage of OPU is the possibility of using over a short time, or even on the same collection (when many oocytes are recovered), more sires to achieve in a short time several different dam-sire combinations. This is especially relevant for those donor cows of high genetics that are requested as sire's dams.

IN VITRO TECHNIQUES AND OOCYTE QUALITY

Following collection the oocyte must undergo the processes of *in vitro* maturation and fertilization to initiate embryo development. A considerable body of knowledge has accumulated over the years on the biology of the bovine oocyte and reliable procedures are available in the literature to mimic the process of bovine oocyte maturation and fertilization *in vitro*. In general over 80% of the immature oocytes transferred in the commonest maturation medium, TCM 199 with 10% FCS and gonadotropins, complete maturation and around 70% of them are successfully fertilised. Following cleavage several different protocols can be employed to develop the early embryos up to the blastocyst stage. Co-culture systems and cell-free systems are used depending on the different laboratories and also an *in vivo* culture system, in the oviduct of sheep temporary recipients, is successful. As recently demonstrated by Lonergan *et al*. (2001), the culture system applied from cleavage to blastocyst is the critical factor that affects embryo quality while, as mentioned above, it is the intrinsic oocyte quality that determines embryo yield. An interesting approach has been developed in the last few years to improve the quality of the oocytes through a prematuration culture period of 24–48 h, in presence of molecules capable of blocking the oocyte in the germinal vesicle stage. This method, generally referred to as two-step culture system, was expected to help the oocyte to complete its cytoplasmic capacitation during the prematuration step therefore improving its ability to undergo the following maturation and to develop into a viable embryo. Unfortunately, no improvement of developmental capacity has been demonstrated in oocytes subjected to prematuration. Interestingly however, this method has proven valuable to manipulate the timing of the maturation process during complicated procedures such as nuclear transfer (Lagutina *et al*., 2002) given that the embryos produced by the two-step culture system have normal viability (Ponderato *et al*., 2002).

CONCLUDING REMARKS

The successful application of the *in vitro* embryo production technology requires a highly trained technical team and a fully equipped assisted reproduction laboratory. It is therefore a demanding technique and consistent success is the result of scientific knowledge, dedication and attention to details. In the future, a more widespread use of assisted reproduction techniques in cattle will be facilitated by continuous support

for basic studies and also by the ability of the cattle industry to fully exploit the opportunities offered by scientific advances.

REFERENCES

Bousquet, D., Twagiramungu, H., Morin, N., Brisson, C., Carboneau, G. and Durocher, J., 1999. *In vitro* embryo production in the cow: an effective alternative to the conventional embryo production approach. *Theriogenology*, **51**(1), 59–70

Butler, W.R., 1998. Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. *J Dairy Sci*., **81**(9), 2533–2539

Galli, C. and Moor, R.M., 1991. Development of immature bovine oocytes into viable embryos *in vitro*. *Bulletin de l'Association des Anatomistes*, **75**(228), 67–71

Galli, C. and Lazzari, G., 1996. Practical aspects of IVM-IVF in cattle. *Animal Reproduction Society*, **42**, 371–379

Galli, C., Crotti, G., Notari, C., Turini, P., Duchi, R. and Lazzari, G., 2001. Embryo production by ovum pick-up from live donors. *Theriogenology*, **55**, 1341–1357

Galli, C., Duchi, R., Crotti, G., Turini, P., Ponderato, N., Colleoni, S., Lagutina, I. and Lazzari, G., 2003. Bovine embryo technologies. *Theriogenology*, **59**, 599–616

Garcia, A. and Salaheddine, M., 1998. Effects of repeated ultrasound-guided transvaginal follicular aspiration on bovine oocyte recovery and subsequent follicular development. *Theriogenology*, **50**(4), 575–585

Hendriksen, P.J.M., Vos, P.L.A.M., Steenweg, W.N.M., Bevers, M.M. and Dieleman, S.J., 2000. Bovine follicular development and its effect on the *in vitro* competence of oocytes. *Theriogenology*, **53**, 11–20

Kenny, D.A., Humpherson, P.G., Leese, H.J., Morris, D.G., Tomos, A.D., Diskin, M.G. and Sreenan, J.M., 2002. Effect of elevated systemic concentrations of ammonia and urea on the metabolite and ionic composition of oviductal fluid in cattle. *Biol. Reprod*., **66**(6), 1797–1804

Lagutina, I., Ponderato, N., Lazzari, G. and Galli, C,. 2002. Kinetics of oocyte maturation and subsequent development of IVF, parthenogenetic, and NT bovine embryos after meiotic inhibition with roscovitine. *Cloning Stem Cells*, **4**(2), 113–119

Lazzari, G., Wrenzycki, C., Herrmann, D., Duchi, R., Kruip, T., Niemann, H. and Galli, C., 2002. Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biol. Reprod*., **67**(3), 767–775

Lonergan, P., Rizos, D., Ward, F. and Boland, M.P., 2001. Factors influencing oocyte and embryo quality in cattle. *Reprod. Nutr. Dev*., **41**(5), 427–437

Ocon, O.M. and Hansen, P.J., 2003. Disruption of bovine oocytes and preimplantation embryos by urea and acidic pH. *Dairy Sci*., **86**(4), 1194–1200

Ponderato, N., Crotti, G., Turini, P., Duchi, R., Galli, C. and Lazzari, G. Embryonic and foetal development of bovine oocytes treated with a combination of butyrolactone I and roscovitine in an enriched medium prior to IVM and IVF. *Mol. Reprod. Dev*., **62**(4), 513–518

Veterinary Research Communications, 28 (2004) 127–135

Buffalo Milk: Its Properties, Dairy Yield and Mozzarella Production

L. Zicarelli
DISCIZIA, Faculty of Veterinary Medicine, "Federico II" University, Naples, Italy
Correspondence: DISCIZIA – Sez. B. Ferrara, Faculty of Veterinary Medicine, Via F. Delpino, 1, 80137, Naples, Italy
E-mail: zicarell@unina.it

The water buffalo (*Bubalus bubalis*) does not yet have such characteristics as to warrant the label "dairy animal". The buffalo bred in Italy produce almost exclusively milk with a good income while buffalo meat has always been considered a by-product and only in recent years is being considered as for co-primary production. The milk of this species accounts for over 50% of drinking milk, in certain developing countries, such as India, Pakistan, Egypt and Nepal, while in Italy buffalo milk is almost exclusively used for mozzarella cheese production.

Since the establishment of a Herd book, the "Italian Mediterranean Buffalo" has shown increasing production (from 1977 to 2002) both in terms of quantity (Fig. 1) and quality (Fig. 2), which may be chiefly attributed to a change in feeding techniques (Table I). Indeed, comparisons between findings in 1967 (Intrieri and Minieri, 1967), 1977 (Zicarelli *et al*., 1977) and 2000 (Potena *et al*., 2001) show that there has been an increase in the fat (Fig. 3) and protein content of milk (Fig. 4) both in the catabolic

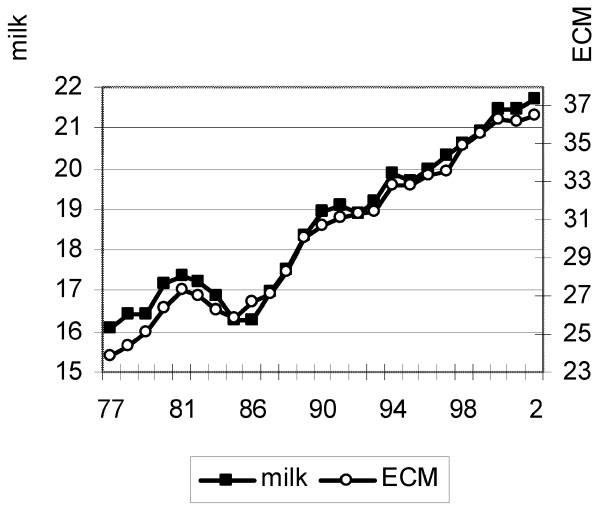


Figure 1. Milk and ECM (740 kcal) production trend between 1977 and 2002

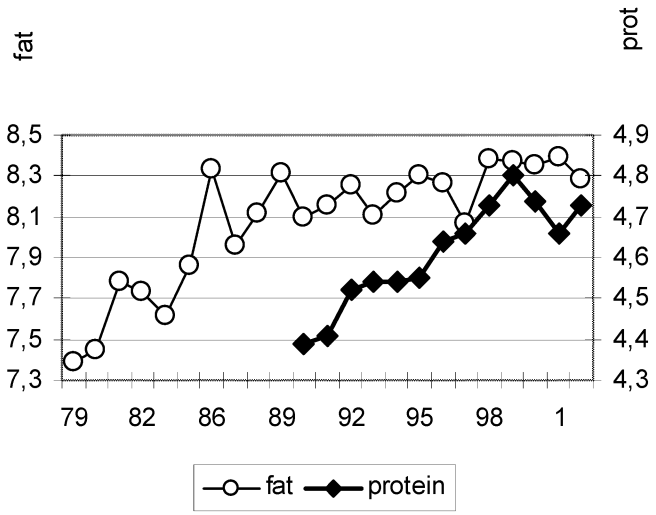


Figure 2. Trend in milk fat and protein (prot.) percentages between 1977 and 2002

TABLE I
Production by lactation of the Italian Mediterranean Buffalo listed in herd book

Year	Milk (kg)	ECM (kg)	Mozzarella cheese yield (%)	Mozzarella (kg)
1977	1.608	2.388	22.9	369
2002	2.168	3.648	26.0	564

and anabolic phases of lactation. This may be attributed to the increase in energy of the diet (0.75 vs 0.9 UFL/kg D.M.) and protein density (11% vs 15%) obtained chiefly with the decrease in the amount of forage (90% vs 45–50%). Part of the increase in protein content occurring in recent years may also stem from the increase in milk urea content, although no correlations have emerged between proteins and milk urea ($r = -0.08$) or between urea and cheese yield ($r = -0.10$).

In recent studies, there was a good correlation between urea (Fig. 6) and milk freezing point – MFP – ($r = 0.47$). The latter result, besides confirming our previous findings (Campanile *et al.*, 1998) which indicated that MFP values may depend on dietary factors (wide NSC/protein ratio and/or low protein values), also showed that the lactation phase (Potena *et al.*, 2001), regardless of the type of diet adopted, may alter both the urea and MFP values. This assumes major importance for buffalo farming as the herd, due to buffalo reproductive seasonality, changes the mean number of days in milk during the year and hence the milk may sometimes show higher values of MFP that do not stem from the addition of water.

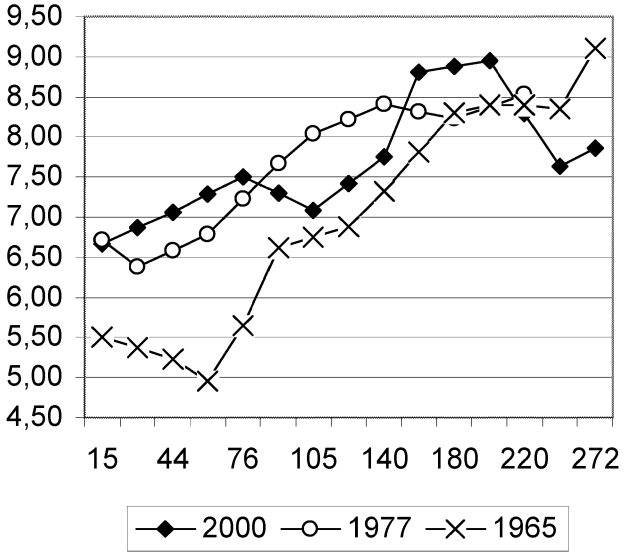


Figure 3. Fat percentage during lactation in buffalo in 1967, 1977 and 2000

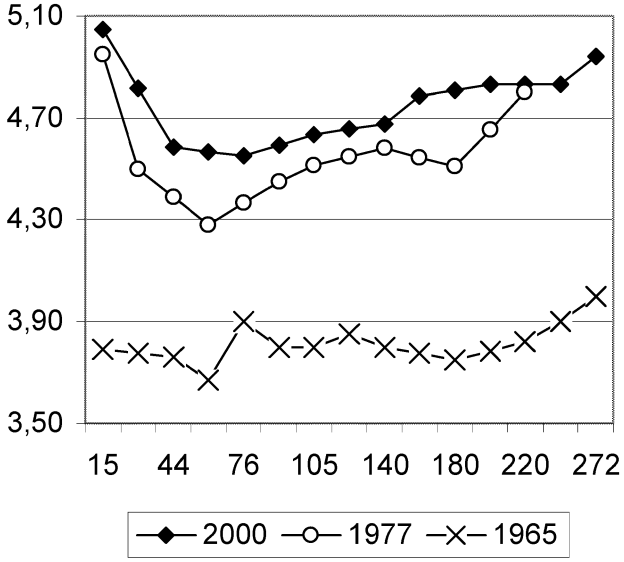


Figure 4. Percentage of protein in milk during lactation observed in buffalo in 1965, 1977 and 2000

Figure 5. Protein percentage (p) and cheese yield (%)

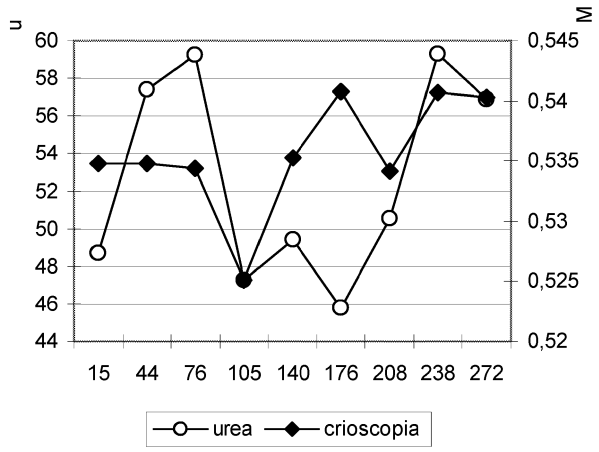


Figure 6. Trend in urea (u) and MFP (M)

The increase in milk quality during recent years has also caused an increase in cheese yield as a significant correlation with milk protein content ($r = 0.47$) has been demonstrated (Fig. 5) in experimental trials (Potena *et al*., 2001).

Compared with cow's milk, buffalo milk has a higher percentage of all components (Table II). In spite of its higher fat percentage, milk and mozzarella cholesterol content is lower for buffalo than for cow's milk (275 mg vs 330 mg and 1562 mg vs 2287 mg respectively). This is of major interest, together with some studies that report

TABLE II
Chemical properties, dairy yield and yield:milk protein ratio of milk adjusted for calorie value (kcal 740) and fat content (4%) of Bruna (B.I.), Friesian (F.I.) and "Italian Mediterranean Buffalo" (B)

	Milk				Adjusted Milk (kcal 740)				Yield % at the same energy content (kcal 740)	
	Fat (%)	Protein (%)	Yield (%)	Yield/ Protein	Fat Protein (%)	Yield (%)	Yield/ (%)	Yield Protein	Yield/ (%)	Protein
BI	4.21	3.55	12.1	3.4	3.83	3.24	11.0	3.4	12.0	3.4
FI	3.44	3.10	9.5	3.1	3.76	3.39	9.8	2.9	9.6	3.1
B	8.30	4.65	25	5.4	4.94	2.77	14.9	5.4	19.6	4.2

a larger number of small fat globules in buffalo milk as compared to bovine and sheep milk. It is well known that small fat globules are rich in polyunsatured fatty acids (Martini *et al*., 2003).

Thanks to its higher dry matter content, buffalo milk has a higher cheese yield that, however, does not seem to be due solely to the different milk quality. For example, for each protein percent point more than 5 cheese yield points are obtained for buffalo and about 3 for cows even when milk is adjusted for the same caloric content. The difference is attenuated although it still persists if the cheese yield is adjusted for fat content (Table II).

Unlike bovines, as shown by the researches of Addeo *et al*. (1977), no polymorphisms have been detected for α_{s1}, β or the κ casein, except for differences in the electrophoretic mobility of α_{s2} casein fraction. The κ casein is similar to the cow's κB casein and has 7 main fractions (Addeo *et al*., 1977), of which k4 and k5 show two more (a and b) and k7 four more (a, b, c and d). Fraction k_1 represents respectively 40% of the total k-casein in buffalo and is very similar to kB_1-casein in the cow, where it accounts for only 25% of total k-casein (Addeo *et al*., 1977). The larger quantity (Table III) of k-casein as compared with the cow speeds up the enzymatic curding phase which requires a smaller quantity of renin. Optimal curd elasticity is obtained for the buffalo at pH 4.9, and for the cow at pH 5.2–5.0 (Addeo *et al*., 1996).

The amino acid composition of the κ-casein of the two species differs in the quantity (mole/mole protein) of N-acetylgalactosamine (0–4.3 and 0–6.7 respectively in buffalo and cow) and sialic acid (5.5–8.5 and 3.5–4.3 in buffalo and cow).

α_s casein is constituted by α_{s1} and α_{s2} fraction. α_{s1} casein does not differ much in the two species and consists of fractions α_{s0} (Petrilli *et al*., 1979), α_{s1}-II and α_{s1}-I which are differentiated respectively by the presence of eight, seven and six phosphate groups. In α_{s1} casein of buffalo, sheep and goats Gly 192 is found while in cows this is

TABLE III
Percentage of different casein fractions in cow and buffalo

	Buffalo (%)	Cow (%)	Buffalo/cow (%)
α_{s1}	30.2	38.4	78.6
α_{s2}	17.6	10.5	167.6
β	33.9	36.5	92.9
k	15.4	12.5	123.2
Total	97.1	97.9	
$\alpha_{s1} + \alpha_{s2}$	47.8	48.9	97.7

replaced by Glu 192. This substitution most likely occurred when *Bos taurus* (Richardson *et al*., 1992) became differentiated from other ruminants. In an alkaline environment the electrophoretic mobility of the three components of buffalo α_{s1} casein is lower than that of bovine. This is a property which enables the presence of cow's milk to be detected (over to 5%) in the event of sophistication (Ferranti *et al*., 1996). Moreover, due to a mutation, there is a phosphorylate residue instead of hydrophobic one, which is responsible for the enhanced non-polar nature of the buffalo protein. In contrast, in cow's milk α_{s1} casein has a phosphoseryl group in position 115 which is surrounded by hydrophobic amino acids. The absence of phosphoserin 115 in the α_{s1} casein of buffalo strengthens the non-polar nature of this protein (Ferranti *et al*., 1998). The interaction of hydrophobic groups is probably responsible for the characteristics of the α_{s1} casein in the context of casein micelles. Finally, the loss of a phosphate group, the increase in density and higher sensitivity of buffalo casein micelles to chymosin may partly explain the shorter clotting time and the higher cheese yield of water buffalo as compared to bovine milk (Addeo *et al*., 1980). The cheese-making potential of buffalo milk is also better than that of the cow due both to the higher casein content and the phosphorylation characteristics of α_{s2} casein: 10 and 11 phosphate groups/mole (in that it consists of two fractions) vs four respectively in buffalo and cow (Ferranti *et al*., 1996; Addeo *et al*., 1996).

Buffalo's β casein is similar to that of the cow and has two variants. Of these, A has been found only in Venezuelan buffalo and differs from B in three amino acids (Ferranti *et al*., 1998). The two variants A and B closely resemble cow's βA_2 casein, differing from the latter by four and five amino acids respectively. The β and α_{s1} casein fractions make up 70% of the micellar network of proteins. Hydrolysis of buffalo β casein with plasmin produces the fractions γ_2 and γ_3. Thanks to the identification of fraction γ_2, it is possible to detect the presence ($<1\%$) of cow's milk in buffalo mozzarella (Addeo *et al*., 1989). Finally, the protein pattern of the two species differs in the smaller quantity of α_{s1} and β found in buffalo, as well as the larger quantity of k-casein and α_{s2} casein (Table III). However, the absence of polymorphisms for α_{s1}, β

and the κ casein, that is found for bovine milk, may suggest that there are no differences between milk of different buffalo populations and/or subjects.

No differences have been found between buffalo and bovine variant B of β-lactoglobulin while some differences exist between the two species in the B variant of α-lactoalbumin. Recently a novel variant of α-lactoalbumin has been found, called A, that differs from the analogous bovine A variant in that it seems to contain less glucides. The frequency of the AA genotype is very low, about 0.5% as is the AB one (2.4%) (Chianese *et al*., 2001).

In a recent trial carried out over two consecutive years (Zicarelli *et al*., 2001), it emerged that by using the formula of Altiero *et al*. (1989), which calculates cheese yield by taking milk fat and protein content into account, it is possible to identify buffalo (Table IV) which, given the same calculated cheese yield and hence the same milk lipoprotein content, have a different experimental cheese yield. For these buffaloes it has been calculated that one point more of milk protein percentage supplies 57 g of curd for buffaloes with an experimental cheese yield higher than that calculated and 53 g for those in which the experimental cheese yield is lower than the

TABLE IV
Individual calculated and actual yields according to the latter and to milk production

	Calculated yield (g/kg)			Clot weight		Δ clot weight – calculated		Clot weight – calculated yield adjusted for urea	
Groups	Yield	Adjusted for urea	Δ	at 4 h	at 24 h	at 4 h	at 24 h	at 4 h	at 24 h
YEAR 1 – Actual cheese yield									
High	253	246	7	273^{A}	242^{A}	20^{A}	-9^{A}	27^{A}	-4^{A}
Low	251	244	7	254^{B}	230^{B}	3^{B}	-21^{B}	10^{B}	-14^{B}
Production									
>30(q)	249	244	5	253^{a}	229^{a}	4^{a}	-20^{a}	9^{a}	−15
<30(q)	253	246	7	267^{b}	239^{b}	14^{b}	-14^{b}	21^{b}	−7
YEAR 2 – Actual cheese yield									
High	268^{A}	261^{A}	7	286^{A}	266^{A}	18	−2	25	−5
Low	247^{B}	235^{B}	12	256^{B}	239^{B}	9	−8	21	−4
Production									
>30(q)	252	248	4	265	246	13	−6	13	−2
<30(q)	254	251	3	268	251	14	−3	17	0
Between years									
Year 1	251	248	3	272	253	21^{a}	2^{a}	24	-5^{a}
Year 2	258	252	6	263	236	5^{b}	-22^{b}	11	-16^{b}

calculated one. Buffaloes with the higher yield were those that had produced less milk per lactation (< 3000 kg) in the first year of the trial.

In the second year, buffaloes were monitored between 130 and 160 days of lactation, insofar as the yield in this phase had correlated strongly ($r = 0.93$, $p < 0.001$) with the average cheese yield over the whole period of lactation. In this trial, no differences emerged concerning the cheese yield among the animals that had produced more or less than 3000 kg of milk/lactation, while buffaloes with different experimental cheese yield also showed a different value for theoretical cheese yield. A higher value of experimental cheese yield was found for 3 out of 14 (21.4%) of buffaloes yielding more than 3000 kg of milk and 18 out of 33 (54.5%) of buffaloes yielding less than 3000 kg of milk per lactation.

In the first year no differences emerged in the rheological parameters between buffaloes with high and low experimental cheese yields. By contrast, the more productive animals (more than 3000 kg of milk) showed a shorter enzymatic phase and higher SH values over the two years. No relationship was found between milk characteristics over the two years of the trial, while the cheese yield in the fist year was related to the cheese yield at 4 ($r = 0.765$; $p < 0.01$) and 24 h ($r = 0.732$; $p < 0.05$) from milking, in the second year.

Further studies are required to identify buffaloes whose casein retains more whey and more fat during the clotting process and to ascertain whether there are protein fractions associated with the phenomenon. Moreover, also with respect to the dairy cow, the genetic variants detected up to now do not fully justify the differences in yield. Recently we ascertained (in press) that Bruna Italiana cows have a higher milk yield than Italian Friesians, having the same k-casein variant and milk fat content.

This is particularly important in that a higher cheese yield is also synonymous with an enhanced cheese-making potential and hence with milk able to provide cheese without recourse to particular technological stratagems. This feature has enabled the "Mozzarella di Bufala Campana" DOP (MBC) to achieve its well-known success: of the DOP cheeses it ranks third according to sales which have continually risen since the establishment of the MBC consortium. Albeit difficult to achieve, selection should aim to identify those higher-cheese-yielding animals which are not always those that produce milk with a higher protein content. By selecting only for higher milk production, one risks increasing the number of animals within the buffalo population producing milk which is not always optimal for processing.

Selection made for higher-cheese-yielding animals is even more likely to characterize the "Italian Mediterranean Buffalo", since it cannot be done with the genetic variants of milk. This would further validate the existing regulations of MBC that restrict the product to exclusive processing of milk from the "Italian Mediterranean Buffalo" and that pursue the aim of binding the product to the breed and the geographical area. The reference to a link with a certain geographic area is represented by the use of local forage for animal feeding and is greatly emphasized by the amendment to the MBC production regulation regarding milk processing, which stipulates that "acidification of milk must be achieved by adding whey, derived from

previous buffalo milk processing". This technique does not enhance product shelf-life but is a characteristic which has distinguished MBC from other stretched curd cheeses and has underpinned its success. If changes are to be made, they should aim to increase the frequency of supply to large distributors as this would even further guarantee the label "fresh product".

REFERENCES

Addeo, F., Chobert, J.M. and Ribadeau-Dumas, B., 1977. Fractionation of whole casein on hydroxyapatite. Application to a study of buffalo x-casein. *Journal of Dairy Research*, **44**, 63–68

Addeo, F., Mercier, J.C. and Ribadeau-Dumas, B., 1977. The casein of buffalo milk. *Journal of Dairy Research*, **44**, 455–468

Addeo, F., Pelissier, J.P. and Chianese, L., 1980. *Journal of Dairy Resarch*, **47**, 421–426

Addeo, F., Moio, L., Chianese, L. and Nota, G., 1989. Evaluation of bovine and water buffalo milk in mixtures of liquid milk and mozzarella cheese by gel isoelectric focusing. *Italian Journal Food Science*, **3**, 71–80

Addeo, F., Emaldi, G. and Masi, P., 1996. Tradition and innovation in the "Mozzarella di Bufala Campana cheese" production. *Proceedings of the International Symposium on Buffalo Products*, Wageningen Press, The Netherlands, 23–40

Altiero, V., Moio, L. and Addeo, F., 1989. Previsione della resa in mozzarella sulla base del contenuto in grasso e proteine del latte di bufala. *Scienza e tecnica lattiero-casearia*, **40**, 425–433

Campanile, G., De Filippo, C., Di Palo, R., Taccone, W. and Zicarelli, L., 1998. Influence of dietary protein on urea levels in blood and milk of buffalo cows. *Livestock Production Science*, **55**, 135–143

Chianese, L., Caira, S., Pizzolongo, F., Ferranti, P., Pugliano, G. and Addeo, F., 2001. Primary structure of water buffalo milk: α-Lactoalbumin variants A and B. *Proceedings of VI World Buffalo Congress*, Caracas, Venezuela, **II**, 410–419

Ferranti, P., Chianese, L. and Addeo, F., 1996. The primary structure characteristics of the buffalo caseins and analytical applications for the "Mozzarella di Bufala Campana cheese" genuiness defense. *Proceedings of the International Symposium on Buffalo Products*, Wageningen Press, The Netherlands, 187–191

Ferranti, P., Scaloni, A., Caira, S., Chianese, L., Malorni, A. and Addeo, F., 1998. The primary structure of Water Buffalo α_{s1} and β-casein: Identification of phosphorylation sites and characterization of a novel β-casein variant. *Journal of Protein Chemistry*, **17**, 8

Intrieri, F. and Minieri, L., 1967. Sull'andamento del valore dei costituenti chimici del latte di bufale del casertano in rapporto alla distanza dal parto. *Acta Medica Veterinaria*, **XIII**, 3–4, 240–249

Martini, M., Spinelli, S., Scolozzi, C. and Cecchi, F., 2003. Studio sulle caratteristiche lipidiche del latte di bufale allevate in Toscana: nota II. *Atti II Congresso nazionale sull'allevamento del Bufalo*, 28–30 Agosto, 2003, 147–151

Petrilli, P., Chianese, L. and Addeo, F., 1979. Buffalo α_{s0}-casein: isolation and characterization. *Journal of Dairy Research*, **46**, 231–233

Potena, A., Bove, D., Cocca, T., Haubner, T. and Zicarelli, L., 2001. Andamento di alcune componenti del latte di bufala in funzione della distanza dal parto: risultati preliminari. *Atti I Congresso Nazionale sull'allevamento del bufalo*, 3–5 ottobre, Eboli, 231–235

Potena, A., 2002. Caratteristiche chimico fisiche e resa del latte di bufala, PhD Thesis XV cycle, pag. 1–138

Richardson, T., Oh, S., Jimenez-Florez, R., Kumasinski, T.F., Brown, E.M. and Farrel, H.M. Jr., 1992. Advanced Dairy Chemistry – *I Proteins* (Fox, P.F., editor), Elsevier, London, 545–578

Zicarelli, L., Piccolo, V., Barbieri, V. and Di Lella, T., 1977. Effetti della doppia mungitura meccanica nella bufala. *Atti Soc. It. Sci. Vet*., Camerino, 658–659

Zicarelli, L., Potena, A., De Filippo, C., Di Palo, R. and Campanile G., 2001. Confronto tra bufale con differente resa alla caseificazione. *Atti I Congresso Nazionale sull'allevamento del bufalo*, 3–5 ottobre, Eboli, 241–246

Veterinary Research Communications, 28 (2004) 137–142

Economic Problems in the Buffalo Milk *filière*

F. de Stefano
Dipartimento di Economia e Politica Agraria, Università di Napoli Federico II, via Università 96, 60055 Portici (NA),Italy
Correspondence: Dipartimento di Economia e Politica Agraria, Università di Napoli Federico II, via Università 96, 60055 Portici (NA), Italy
E-mail: destefano@unina.it

INTRODUCTION

To avoid any confusion, it should be pointed out that this paper will examine the question:

(a) of buffalo milk and mozzarella, but not that of Buffalo Mozzarella from Campania (*Mozzarella di Bufala Campana*) nor that of cow's milk, unless expressly indicated;
(b) of the buffalo in its entirety, despite dealing separately with its components.

The discussion will necessarily be brief, albeit with references to the main issues of consumption, processing, distribution and milk production, as well as general prospects.

1. Trend in buffalo mozzarella consumption

According to ISMEA, continuing the trend of previous years, in 2000 domestic consumption of buffalo products rose further, although the same year saw a contraction across the board in the consumption of other DOP cheeses, as occurred across the whole cheese sector. By contrast, demand for DOP cheeses in 2001 showed no variations in volume, although it increased in value by 5.5%. In particular, Mozzarella di Bufala Campana experienced increases, for 2001, both in quantity (5.9%) and in value (12.4%) (Fabbro, 2003; Osservatorio Consumi, 2003). These consumption trends in buffalo products occurred within a price framework which is on average 50% higher than that of mozzarella made from cow's milk.

Consumption is chiefly located in Southern Italy. However, there seems to be a clear trend towards greater distribution in other parts of Italy. In other words, despite being more concentrated in the areas where the raw material is produced, buffalo mozzarella consumption is spreading throughout Italy and abroad to the same extent as other, longer-established products. In the geographical areas far removed from the production area, percentage variations in the quantities consumed are three times higher in north-western and central Italy than in the south. Finally, it is precisely this

expansion of the buffalo mozzarella market and its greater product penetration which afford the greatest short-term prospects for overall sales expansion, and not the increase in per capita consumption.

2. *Recent trends in processing*

The supply of buffalo milk, and hence that of mozzarella, is also continually expanding, both in terms of quantity and quality. All this suggests generally positive forecasts for sectoral development, although there are still doubts concerning the sector's actual ability to match, or even promote further growth, with appropriate improvements in processing and distribution. Several years ago it was thought "that buffalo mozzarella has a potential market which is perhaps difficult to reach, but whose theoretical size may be valued at about three times that of its current market" (de Stefano, 1999). This forecast still holds, although subject to certain conditions.

The processing of buffalo mozzarella is structurally different from that of cow's milk mozzarella. The dairies are smaller, they use less advanced techniques and usually operate on geographically very limited markets, which often coincide with the production areas of the raw material (de Stefano, 1996). The degree of competition between the dairies is high, the tendency to specialize is low, and investments in the distribution and marketing sector are almost non-existent.

3. *Recent aspects of distribution*

The distribution of buffalo mozzarella has specific features that distinguish it from the rest of the cheese-making sector. These concern the greater importance on total sales

TABLE I
Farms and head of buffalo by province in the DOP area

	1990*		2000**	
Province	Farms	Head	Farms	Head
Caserta	837	46,182	1,002	105,100
Frosinone	477	5,490	456	11,200
Latina	289	9,032	350	14,833
Naples	12	539	28	6,270
Salerno	220	14,803	325	33,095
Benevento			3	510
Rome	8	841	3	520
Total	1,843	76,887	2,167	171,528

Source: *ISTAT; **Consortium for the Protection of Buffalo Mozzarella.

of specialised and traditional firms as compared with large distributors, assisted retail as opposed to self-service, and bulk purchase over packaged purchase, while the distributor's brand name has only recently appeared upon sale of the product (de Stefano *et al*., 2001).

Distribution outside the local market is generally not effected by the dairies, but is entrusted to third parties. In this situation, hardly any dairies have up until now been involved in labelling policies. The product is usually sold in traditional low-hygiene packages of varying weight which are ill-suited to modern requirements of handling and display, especially those of supermarkets. It is thus no surprise that much buffalo mozzarella is sold via traditional assisted retail or that it is limited to the segment of gastronomic products.

One of the factors slowing down the adoption of more modern sales approaches is undoubtedly the limited shelf life of the product, on average shorter than that of the cow's milk product. This is also why the latter has a higher service content, and is marketed to a greater extent in fixed weight packaging.

4. Buffalo milk production

The buffalo farm sector is expanding both in typical areas in Italy and in other areas of central-northern Italy, and even abroad.

Yield expressed in milk is rising, breeding techniques and animal welfare are improving and livestock quality and milk hygiene have also shown considerable signs of improvement (Cerrato, 1999: Flora, 2002; de Stefano, 1999). However, in this period of great progress throughout the farming sector in Europe, the improvements recorded in this specific sector may be described as limited. They chiefly concern the search to contain production costs, while insufficient attention is paid to improving quality. This is particularly applicable to the organoleptic characteristics of milk and even, in some areas, herd welfare and the techniques adopted. For example, improvements introduced to animal diets explicitly aim to reduce production costs, and have little to do with the need to guarantee milk quality.

Economic globalisation is also affecting this sector, which is why buffalo farms in other countries, especially in Europe, are drawing ever closer to Italian and international markets.

5. Market development prospects

Buffalo mozzarella still experiences considerable competition from the cow's milk product, due both to high substitutability between the two products in the consumer market, and to the effects caused by distribution. Thus, in order to assess the prospects of the sector, we should not overlook trends due to changes in distribution channels. Indeed, large-scale distribution was in the past oriented towards a preference for

cow's milk mozzarella, especially due to its greater adaptability to the requirements of modern sales strategies and techniques. Despite attracting a specific consumer segment, buffalo mozzarella ended up occupying market spaces left free by its cow's milk counterpart and by the firms producing it, rather than winning its own independent share. In other words, buffalo mozzarella has seen its own market grow in recent years because the market for the cow's milk product has grown. Expansion of the latter has been mainly due to:

(a) changes in consumption styles and tastes on the part of Italian consumers;
(b) growth in modern distribution and promotion of the cow's milk product by the large cheese-making industries.

Buffalo product sales have grown continuously in the recent past, although to a lesser extent overall than might have been expected, given its favourable reception by consumers. The key reasons for this lie in:

(a) the structural and organisational limits of the industrial processing sector;
(b) the notable shortcomings currently found in the distribution of the end product;
(c) inadequate strategies for improvement of milk quality.

Moreover, the marked growth in the supply of milk and processed cheese in recent years, which should continue in the immediate future, means that the markets for the end product also have to expand at a similar rate. As the wholesale demand elasticity of the end product is not high and the demand elasticity of milk on farms is low, there only needs to be a modest surplus in supply for there to be severe reductions in milk selling prices on the part of farmers.

Thus, in the future the market will play an extremely important role. In particular, the distribution sector will become increasingly demanding with regard to terms and conditions of supply, requiring consistently high quality standards of goods and product safety, and generally all those features that allow the adoption of more modern techniques of packaging, promotion, merchandising, and management (Del Giudice and de Stefano, 2002). Such requirements are ill-matched by a structure which is little more than artisanal, found in most of the buffalo milk processing sector.

The above-mentioned factors, which affected the whole mozzarella market in the past, have ended up almost spontaneously benefiting consumption of the buffalo product as well. These factors could well decelerate in the future. Thus, it is necessary to adopt specific development strategies for the processing of buffalo mozzarella, which should have two different objectives.

(a) On one hand, dairies, once their technology has been upgraded, will have to continue to target precise market niches, in which to offer a consistent, extremely differentiated, easily recognisable high-quality product, with a high degree of added service and perhaps a semi-artisanal appearance.
(b) On the other hand, the more dynamic and ambitious firms will have to explicitly target modern distribution channels and more distant markets, not being

satisfied only with existing niches, but launching themselves to win large market shares, in open competition with the cow's milk product.

Finally, we should especially focus on the relationship that may be established between buffalo mozzarella, intended as a typical product with high development potential, and the modern strategies of large-scale distribution. In general terms, "typical products with high development potential" are those whose production on the one hand shows a non-negligible economic and productive dimension, combined with good market growth prospects, and on the other an organisational level of the filière with considerable limits and shortcomings. This definition is perfectly suited to buffalo mozzarella. To develop such production, stress has been repeatedly laid on the ever-diminishing importance of price-based competitive strategies and the growing importance of strategies based on improvement and diversification of services and assortment.

Increasingly, therefore, the operative and strategic choices of the large Italian and European distributors will be translated into an increase in available customer services, the creation of a more direct relationship between employees and consumers, and into planning a product range guided by new criteria. Of these, the regional product range is assuming considerable importance.

Therefore, taking due account of further growth in the importance of large distribution and hence the greater importance of the rationale of modern trade, the sectors producing typical foodstuffs in traditional manner have to rethink their own strategic choices so as to take advantage of the chances offered by the new strategies of large distributors. This opens up even more needs and opportunities for market development of buffalo mozzarella, which will need to be taken into account in the short term.

CONCLUSION

The situation briefly outlined above indicates many concluding considerations. Without wishing to list them one by one, it would be worth focusing on at least two major points. These considerations also dictate specific strategies for the buffalo milk filière, both for the distribution sector and for farms.

The first point is that buffalo mozzarella differs from most of the other typical products in Italy, especially because:

(a) it has already reached high levels of supply and demand;
(b) its supply continues to increase sharply.

In other words, buffalo mozzarella can no longer be considered a niche product. If we wish to seek a parallel, then we should be careful not to commit the mistake typically made by many parents, who fail to realise that their children have grown up, and continue to treat them as children even when they have become adults! Going back to buffalo mozzarella, it should be realised that traditional markets, although these

should not be neglected, may well have become insufficient to absorb the foreseeable future increase in supply, and that wider markets must therefore be conquered. This means that the increase in distribution must necessarily pass through large distributors, something that in turn will force the processed product to learn to adapt to the many technical and organisational requirements of these distributors, without relinquishing its typical organoleptic characteristics. It is chiefly up to the industrial processing sector and scientific and technological research to ensure that this fundamental and urgent task is accomplished.

The second point is that the typical nature of Campanian Buffalo Mozzarella is, all things considered, an attribute that could also prove somewhat transient. Such *typicality*, apart from the origin of the raw material, currently lies almost exclusively in the fact that the product is obtained from buffalo milk, irrespective of its other technical and organoleptic characteristics. Yet with the increase in end demand for the finished product, the expansion of buffalo farming into other areas in Italy and the rest of Europe, the increase in interest from multinational economic lobbies in the cheese-making sector, along with the intensification of globalisation processes and their relative market "philosophy", the current typicity of buffalo mozzarella could become increasingly difficult to uphold.

The product therefore needs to also become differentiated by other traits, to be linked to the production area and to farming techniques. In this regard, the organoleptic traits of mozzarella play a fundamental role. Of particular importance is the quality of the raw material. This means that the focus must be on livestock technology, especially diet, perhaps by the rediscovery and diffusion of techniques which are traditional to typical buffalo farm areas and have recently been overlooked as they were unsustainable in terms of production costs. This time the task falls chiefly on the farming sector and once again the research sector.

The economic situation of the buffalo filière is still favourable, although clouds are already gathering on the horizon. There is no reason to be complacent and let the chance to set up the sector's future pass by, especially with the likely prospect of more difficult times just around the corner.

REFERENCES

Cerrato, M., 1999. La filiera della Mozzarella di Bufala Campana nell'area della Denominazione di Origine Protetta. *Bubalus Bubalis*

de Stefano, F., 1996. Market situation for buffalo milk products, in: *FAO, International symposium on buffalo products, EAAP*, **82**, Wageningen Press, Wageningen

de Stefano F., 1999. Ottime potenzialità per l'allevamento bufalino in Italia. *L'Informatore Agrario*, **51**

de Stefano, F. and Del Giudice, T., 2001. Sviluppo del mercato della Mozzarella di Bufala. *Proceedings I Congresso Nazionale sull'Allevamento del Bufalo*, Eboli, Italia

Del Giudice, T. and de Stefano, F., 2002. A new role for typical products in modern retails. *ISSEI Conference Proceedings 2002*, University of Wales, Aberystwyth 22–27 July

Fabbro, D., 2003. Il brand è d'obbligo. *Food*, **1**

Flora, A., 2002. La filiera della mozzarella di bufala: un laboratorio dello sviluppo locale. *La Questione Agraria*, **1**

Osservatorio Consumi, 2003. La spesa domestica per i prodotti tipici nel 2001. *ISMEA*

Veterinary Research Communications, 28 (2004) 143–148

Buffalo Meat Production: Performance *infra vitam* and Quality of Meat

F. Infascelli[1]*, S. Gigli[2] and G. Campanile[1]
[1] *Department of Zootechnic Science and Food Inspection, section B. Ferrara, Faculty of Veterinary Medicine, University of Naples "Federico II", via F. Delpino, 1, 80137 Naples, Italy;* [2] *Animal Husbandry Research Institute, via Salaria, 31, Monterotondo Scalo (Rome), Italy*
* *Correspondence: Dipartimento di Scienze Zootecniche e Ispezione degli alimenti, sez. B. Ferrara, Facoltà di Medicina Veterinaria, Università di Napoli "Federico II", via F. Delpino, 1, 80137 Naples, Italy*
E-mail: infascel@unina.it

INTRODUCTION

In Italy, commercialization of milk for mozzarella cheese production represents the main income of buffalo breeding. This is a critical point for farm economy. Meat production could represent a good collateral or main activity. In addition, the market interest in buffalo meat has progressively increased due to the negative trend towards bovine meat consumption, which has very recently worsened due to the BSE scare.

In the past much research was carried out in the Veterinary (Ferrara *et al*., 1969) and Agricultural Faculties (Matassino *et al*., 1976) of the University of Naples and at the Istituto Sperimentale per la Zootecnia of Rome (Romita *et al*., 1976) demonstrating both the modality of breeding buffalo as a meat producer and the good nutritional characterics of buffalo meat.

PERFORMANCE *INFRA VITAM*

More recently, Di Lella *et al*. (1998), in a trial aiming to verify the influence of the feeding programme on the growth dynamics of young buffalo bulls, divided 24 seven-days-old buffalo calves according to a 2×2 factorial design: two weaning ages (63 d vs 84 d) and two weaning concentrates (CP 17% vs 14%; starch 37% vs 29.6%, as fed). The calves of groups A and B, weaned at 63 d, received 6 l/head/d of acidified milk replacer (in the ratio of 180 g/l of water) until 42 d. Subsequently, the replacer amount was gradually decreased, whilst administering the same volume. The animals of groups C and D received 8 l/head/d of the same milk replacer, but in the ratio of 140 g/l, until 56 d. Also in this case the amount of replacer was gradually decreased, leaving the volume unchanged. Roughly chopped alfalfa hay and weaning concentrate were available from the fifth week; corn silage was administered starting from 70 d.

After weaning, the animals were fed ad libitum hay and corn silage; the concentrates were administered in the amount of 12 kg/d/group. The weaning age did not influence performance. By contrast, the weaning concentrate strongly affected the growth dynamics in the first 6 months: in this phase the concentrate with higher protein and starch content produced better results. However, in the subsequent period (6–16 months) due to compensative growth, improved performance was obtained using concentrates with less favorable characteristics. These observations suggest that concentrate with higher protein and starch content be administered during weaning, when the calves are destined to be slaughtered at lower weights compared to those reached in this trial (400 kg); otherwise it could be opportune to administer the protein-poor concentrate in order to limit feeding costs.

In order to increase the knowledge about buffalo growth, characterised by periods of very scarce weight gain followed by compensatory phases, Infascelli *et al.* (2001) carried out research on 12 six-month-old male buffalo, equally divided into group A (111.0 ± 6.9 kg LW) and B (116.7 ± 7.6 kg LW). Group A was fed 100 g DM/kg $LW^{0.75}$ of diet (CP/DM 14.1%, UFV/kg DM 0.84) based on rye-grass silage, rye-grass hay and concentrate. Group B received the same diet, but every 2 months the DM was alternatively 20% more or less than group A. From 300 kg LW until the end of trial (477 d of age), group B received diet (CP/DM 14%, 0.88 UFV/kg DM) obtained by varying the proportions of feeds. Between 180–238 d of age, group B showed a higher daily weight gain (DWG: g 480 ± 0.46 *vs* 510 ± 0.34, for A and B); however the conversion index (CI) was worse due to the higher amount of DM and UFV intake. In the interval 238–303 d, with a decreasing DM intake, group B showed lower DWG and worse CI. In the following interval, notwithstanding the increase in the DM intake, group B had similar results. On the contrary, between 380–415 d, group B had more favourable CI (UFV/kg weight gain 5.195 *vs* 4.452 for A and B) with slightly lower DWG (g 1143.0 ± 0.28 *vs* 1023.0 ± 0.14, for A and B). Group A showed the best results for the period 180–415 d, even if differences were not significant. Increasing the energy density of the diet in the interval 415–477 d slightly improved the total (180–477 d) DWG of group B, however less than that of group A (855.2 ± 0.58 *vs* 769 ± 0.25). Consequently, the total CI of group B was worse than that of the interval 180–415 d. Considering the whole trial, the buffalo growth curve showed a progressive increase in DWG, reaching its peak at 303 d of age (180 kg LW) until 415 d (300 kg LW). Successively the CI considerably worsened.

An innovative method to obtain "biologic" young buffalo bulls could be the cow-calves system adopted in Brazil (Campanile *et al.*, 2001). As reported in Table I, due to the high price of buffalo milk, the utilization of dairy cow at the end of her productive career to wean two buffalo calves should be financially more favourable. This should produce economically more favourable results also compared with the administration of milk replacers, thanks to the compensation for the nurse by the EU. Because of sanitary reasons (inter-species transmission of infective diseases) the system should be utilised in stalls outside dairy farms.

TABLE I
Costs (€) of production of young buffalo bulls (350– kg LW) weaned with buffalo milk, milk replacer or cow as nurse

	Buffalo milk	Milk replacer	Cow nurse
Milking phase			
€/kg	1.18	1.86	
Milk kg/kg DWG	5.17	1.875 (DM)	
Increment (kg)	200	40	200
Costs (€)			
DWG kg	6.15	3.49	
Feeding	1229.17	139.44	
Feeding nurse/day			3.1
Feeding nurse/day/2 calves			1.55
Feeding nurse/day for 2 calves for 200 days			309.87
Phase 40–350 kg LW			
Total feeding	1379.23	466.44	459.94
Total feeding/0.7*	1970.33	666.34	657.05
Total + slaughtering costs	2073.62	769.63	760.35
Nurse (net final sale)			154.94
Nurse (net final sale/bred calf)			77.47
Feeding + slaughtering + calf bred by nurse			837.81
Meat (kg)	14.87	5.52	6.01
Live weight (kg)	5.92	2.20	2.39
Compenses			
Nurse/2 calves			116.00
Male + slaughtering	237.99	237.99	237.99
Total compenses	237.99	237.99	353.99
Costs			
Total (net of compenses)	1835.63	531.64	483.82
kg of meat	13.16	3.81	3.47
kg of live weight	5.92	1.52	1.38

*Considering the incidence of feeding on the management costs as 70%.

MEAT QUALITY

Buffalo meat shows good quality characteristics according to the usual estimation parameters. As examples we present the data concerning two animal ages connected to different commercial typologies: veal (6 months) and young bull (15 months). Carcass conformation (Table II) is little influenced by animals age, in fact it only changes from 2+ to 3−, while fatness shows a big increase (from 2 to 3−): these data

TABLE II
Carcass evaluation

Age (months)	Conformation	Fatness
6	2+	2
15	3−	3−

are higher than those obtained for bovines (Failla *et al*., 2001). Fat content and dry matter percentage (Table III) increase for aged buffaloes (+2.1 and +2.8 percent respectively) while the protein and ash percentages do not change very much (Cutrignelli *et al*., 1996; Failla *et al*., 2001).

All colour parameters decrease with age so the young buffalo bulls show a darker coloured meat, with a hue closer to magenta, but with less intensity (Table IV). The measured tenderness (as Warner Bratzle shear force) on cooked meat, which is the best parameter for consumer appreciation, does not show differences according to age. The water holding capacity of raw meat, that strongly affects the choice of consumer at the moment of sale, is better for veal, while the difference is not important for cooked meat (Failla *et al*., 2001).

The fatty acid composition (Table V) is significantly different between two typologies. The aged animals show more saturated fatty acids, in particular C 16 and C 18, more C 18:1 and less C 18:2. All the data presented confirm that buffaloes have good meat quality characteristics both at six and at 15 months of age, in comparison with bovines (Gigli *et al*., 1978; Cutrignelli *et al*., 1996). In addition, the difference between

TABLE III
Chemical analysis

Age (months)	Dry matter	Protein	Fat	Ash
6	23.0	20.4	1.5	1.1
15	25.8	21.2	3.6	1.0

TABLE IV
Colour, tenderness and WHC (on LT muscle)

	Colour			Tenderness kg/cm^2	WHC %	
Age (months)	L	H	C	Cooked	Raw	Cooked
6	43.34	37.70	21.04	1.63	0.99	26.70
15	39.15	27.10	18.88	1.67	1.19	26.95

TABLE V
Fatty acids %

Age (months)	6	15
C 16	17.7	21.6
C 18	14.9	18.4
C 18:1	39.1	43.3
C 18:2	22.0	5.6
Saturated	37.5	43.7
Unsaturated	61.5	55.3
Saturated/Unsaturated	0.61	0.79

two typologies of meat is not dramatic so it is possible to utilise both age categories, or other intermediate ones, to supply good meat to consumers.

CONCLUSION

In spite of the higher costs of buffalo meat production, due to their slower growth compared to specialised bovine breds, there are sound reasons to exploit buffalo meat. Its very good nutritional characteristics and the low number of subjects (considering a mean intercalving period of 400 days, the 120,000 cows actually bred in Italy could produce 96,000 calves of which 52% are males) are well appreciated by the consumer from a dietetic point of view as well as the fact that the sector is more controllable, in terms of food security. In our opinion, in order to further promote buffalo meat production and safeguard the consumer, a regulation providing only commercialization of subjects grown in the range of physiological weight gain should be prepared. The animal register of births could represent a good way of verifying if the slaughtering weight agrees with the aforementioned weight gains. The birth concentration in 6–7 months (using or not the out-of-breeding-mating strategy) could cause a discontinuous availability of meat on the market. This problem could be solved by slaughtering the subjects at different ages and modulating growth through the adoption of proper rationing schemes. In this case, the contemporary presence of animals with different ages should be taken into account planning the stalls in order to guarantee a good available space/head.

REFERENCES

Campanile, G., Infascelli, F. and Zicarelli, L., 2001. Considerazioni sulla produzione del vitellone bufalino da carne. *Agrisole*, suppl. II, 23–28

Cutrignelli, M.I., Calabrò, S., Laudadio, P., Grasso F. and Di Lella, T., 1996. Chemical-nutritional characteristics of meat produced by young buffalo bulls. *Proceeding of the XXXI International Symposium of Zoothecny "Role of animal products on animal health"*, Milano, Italy, 13 September, 1996, pp. 101–106

Di Lella, T., Cutrignelli, M.I., Calabrò, S. and Infascelli, F., 1998. Influence of the feeding programme on growth dynamics of buffalo young bulls until 16 months of age. *Bubalus bubalis*, **2**, 81–90

Failla, S., Settineri, D., Bisegna, V. and Mormile, M., 2001. Produzione del vitello bufalino a carne bianca alimentato con due diversi tipi di latte. Nota II: qualità della carne. *Proceedings of the 1st Italian Congress on Buffalo breeding*, Eboli, Italy, 3–5 October, 2001, pp. 281–284

Ferrara, B., Minieri, L., de Franciscis, G. and Intrieri, F., 1969. La produzione della carne bufalina in Italia. *Acta Medica Veterinaria*, **2**, 313–366

Gigli, S., Romita, A. and Borghese, A., 1978. Prove comparative fra vitelli bovini e bufalini. V. Composizione acidica del grasso di varia localizzazione in animali di 28 settimane di età. *Annali dell'Istituto Sperimentale per la Zootecnia*, **11**, 41.56

Infascelli, F., Cutrignelli, M.I., Sarubbi, F. and Campagna, M., 2001. Influence of different rationing scheme on the growth performance of young buffalo bulls. *Proceedings of the VI World Buffalo Congress*, Maracaibo, Zulia, Venezuela, 20–23 May, 2001, pp. 512–518

Matassino, D., Romita, A., Colatruglio, P. and Bordi, A., 1976. Studio comparativo fra bufali e bovini sulla qualità della carne. I. caratteristiche mioreologiche all'età di 20 settimane. *Proceedings of the II National Congress A.S.P.A.*, Bari, Italy, 17–20 May, pp. 373–379

Romita, A., Borghese, A., De Maria, C. and Gigli, S., 1976. Prove comparative di allevamento di bovini e bufali fino a 20 settimane di età. II. Composizione acidica del grasso, prove di assaggio delle carni. *Proceedings of the II National Congress A.S.P.A.*, Bari, Italy, 17–20 May, pp. 363–371

Veterinary Research Communications, 28 (2004) 149–151

Factors Influencing the Quality of Life of the Cat in its Relationship with Owners

S. Adamelli[1]*, L. Marinelli[1], S. Normando[1], A. Camperio Ciani[2] and G. Bono[1]
[1] *Dip. di Scienze Sperimentali Veterinarie;* [2] *Dip. di Psicologia Generale, Università di Padova, Italy*
* *Correspondence: Dip. Di Scienze Sperimentali Veterinarie, Viale dell'Università, 16–35020 Legnaro (PD), Italy*
E-mail: serena.adamelli@unipd.it

Keywords: cat-owner relationship, quality of life, behaviour

INTRODUCTION

In a multiple study between veterinarians and psychologists the owner–pet relationship was evaluated, focusing attention on the problems and the benefits due to the relationship (Bono, 2000; Camperio Ciani 2000; Bono *et al*., 2003) and a multiple approach method was validated to assess the quality of life of the pet in its relationship with the owner (Marinelli *et al*., 2001). The aim of this study was to analyse the relationship between cats and their owners and identify factors that influence the quality of life of the cat.

MATERIALS AND METHODS

Participants were recruited as volunteers from the staff of the University of Padua and their relatives. Cats younger than six months of age and cats that did not live with their owners were excluded from the experiment. The cat–owner relationship was assessed using a multiple approach: four questionnaires and a simple physical examination (Marinelli *et al*., 2001). The questionnaires investigated the owner's features (age, gender, education, marital status, job, features of the family, place of living, size of dwelling and social relations); the cat's features (age, gender, breed, neutered or spayed, age of adoption, source, whether it is living with other animals or not); the care given to the cat and the cat's behaviour (attachment to the owner, house soiling, behaviour towards strangers, cats and other animals). A veterinarian evaluated the cat's physical condition assessing only the nutritional status and ear condition, because in the previous study they were found to be objective indexes of physical condition. The aspects of the physical examination and the answers to the questionnaires regarding care and behaviour were codified on a score scale. The sum of the scores (total score) represented the index of the aspects considered (care given to the cat, cat behaviour and physical condition). The total scores were subjected to

Spearman's Test to determine the correlation with the features both of the owner and the cat.

RESULTS

A sample of 65 owners and 114 cats was analysed. 84.6% of the people were women. In 49.2% of the cases the age ranged from 21 to 30 years. 37% of the owners had degrees and 55.3% were employed. Most of them lived in the city (63%) and had had previous experience with a cat or a dog (89.2%). Most of the cats were cross breeds (95%). 60.5% were females (88.4% spayed) and 39.5% males (62.2% castrated). The cats, acquired in 22% of the cases in the most suitable period of their life (7th–10th week), had been given by friends (33.3%) or had been found by chance (43.8%). They lived with other pets (74.5% with cats and 27% with dogs) and 57.8% of them had had previous diseases. The results of statistical analysis showed that the total scores of the care given to the cats, the cat behaviour and the cat's physical condition correlated with the features both of the owner and the cat (Table I). The total score of the care given to the cats correlated positively with the sex (female) and the education of the owner and with the neutering of the cat, and negatively with the position of

TABLE I
Results of Spearman's Test. Correlation between total scores and features both of the owner and the cat. Only features which correlated are reported in the table

Features	Care	Cat behaviour	Physical condition
Owner			
Age	–	0.32**	–
Gender (female)	0.23*	0.18*	–
Education	0.33*	–	–
Position (student)	−0.18*	–	–
No. of family members	–	−0.18*	–
Presence of children	–	0.34*	–
No. of social activities	–	0.31**	–
No. of affective bonds	–	–	0.26*
All the family look after the cat	–	0.29*	–
Cat			
Age	–	–	−0.19*
Gonadectomy	0.23*	–	−0.19*
Given by friend or relatives	–	–	−0.19*
Cohabitation with other cats	–	−0.32*	–
Cohabitation with other animals	–	−0.19*	–

* $p < 0.05$; ** $p < 0.01$.

student. The total score of cat behaviour correlated positively with: old age and the sex (female) of the owner, the presence of children in the house, participation in organised social activities and care given by all family members, while it correlated negatively with the number of family members and the presence of other animals in the house. The total score of the physical examination correlated positively with the number of affective bonds and negatively with the age of the cat, its origin (given by friends) and neutering.

CONCLUSION AND DISCUSSION

The main obstacle we encountered in carrying out this study was that of finding a representative sample of volunteers. This was due to the need to respect people's privacy and also to the availability only of people sensitive to and very interested in this topic. The results showed that some features both of the owner and of the cat influence the quality of life of the cat. In particular, care given to the cat seems to depend mainly on the cultural and economic situation of the owner (factors such as education and employment position). Cat behaviour seems to be affected not only by its own social environment, but also by that of its owner (social activities, number of family members, presence of children and of other animals in the house). Physical condition does not appear to depend on specific aspects. This is due to the fact that physical condition is influenced by many factors which are not exclusively linked to the cat-owner relationship.

ACKNOWLEDGEMENTS

The authors thank Martina De Zanche for her assistance with data collection and all the volunteers who took part in the study.

REFERENCES

Bono, G., 2001. I problemi relazionali della convivenza tra animale da compagnia e proprietario. In: *Il contributo della Fisiologia al miglioramento della convivenza tra animale e uomo*. Ed. Società Italiana di Fisiologia Veterinaria, 15–27

Bono, G., Marinelli, L., Martini, M., Sancin, S. and Camperio Ciani, A., 2003. La relazione cane-proprietario: Indagine nel contesto urbano e rurale di Padova. *Obiettivi & Documenti Veterinari,* **1**, 55–62

Camperio Ciani, A., 2001. Pet Therapy: terapia o arricchimento ambientale? In: *Il contributo della Fisiologia al miglioramento della convivenza tra animale e uomo*. Ed. Società Italiana di Fisiologia Veterinaria, 1–8

Marinelli, L., Adamelli, S., Simontacchi, C., Gabai, G., Camperio Ciani, A. and Bono, G.,Metodi per la valutazione della qualità della vita dell'animale da compagnia nella relazione con il proprietario. *Atti Società Italiana di Fisiologia Veterinaria*, **IV**, 25–27

Veterinary Research Communications, 28 (2004) 153–155

Urinary Incontinence After Spaying in the Bitch: Incidence and Oestrogen-therapy

A. Angioletti[1]*, I. De Francesco[2], M. Vergottini[1] and M.L. Battocchio[1]
[1,2] *Department of Veterinary Clinical Science;* [1] *Section of Veterinary Obstetrics and Gynecology;* [2] *Section of Veterinary Surgery and Radiology, Faculty of Veterinary Medicine, University of Milan, Italy*
* *Correspondence: Dipartimento di Scienze Cliniche Veterinarie – Sezione di Clinica Ostetrica e Ginecologica Veterinaria – Facoltà di Medicina Veterinaria, Via Celoria, 10, Milano, Italy*
E-mail:andrea.angioletti@tiscali.it

Keywords: urinary incontinence, spaying, oestrogens, bitch

INTRODUCTION

Urinary incontinence is defined as unconscious urine loss during filling, the persistence of which could result in cutaneous disease and lower urinary tract infection for the bitch, in addition to causing sanitary and management problems for the owner. Spaying is one of the various mechanisms involved in urinary incontinence in the canine species (Gregory, 1994; Holt *et al*., 1993). Among surgical (colposuspension, cystourethropexy, collagen or Teflon® prosthesis) or pharmacological (α-adrenergic agonists, oestrogens) treatments, we focused our attention on oestrogen replacement therapy using conjugated oestrogens. Literature suggests that oestrogens are able to improve urethral closure by the presence of specific local receptors able to modulate the number and sensitivity of alpha-2 receptors in the distal urinary tract (Creed, 1983; Miodrag *et al*., 1988). In our work we investigate the incidence of urinary incontinence related to spaying in the bitch and evaluate the efficacy of oestrogen replacement therapy.

MATERIALS AND METHODS

Data are for the years between 1995 and 2001, and include 430 bitches of different ages, breeds and sizes. Follow ups were made every three months after ovariectomy or ovariohysterectomy to monitor the effect of spaying. Acquired incompetence of the ureteral sphincter was diagnosed after eliminating every other possible aetiology by means of diagnostic investigation (abdominal palpation, colposcopy, radiography, echography, urine analysis, etc. …). The owners were advised about the possible side-effects of long-term administration of oestrogens and about the importance of continuous medical treatment. A daily onset dosage able to block urinary incontinence

was found by the administration of natural oestrogens at different concentrations. After two weeks at the onset dosage with no urinary incontinence observed, we passed to a maintenance dosage, eventually reducing the frequency of administration. The bitches with no symptoms were examined once every three months and blood haemocytometric and biochemical analyses were performed to check bone marrow depression and hepatic function. The data concerning the type of surgery performed, the size and the body weight of the bitches were analysed by the use of a chi-square test and odds-ratio to evaluate their possibile correlation with urinary incontinence.

RESULTS

22 of 430 subjects observed (5.1%) presented symptoms of urinary incontinence, with a variable onset period between 1 to 60 months after surgery. Incidence was 72.8% within 12 months. 7 of 201 ovariectomized bitches and 15 of 229 ovariohysterectomized bitches presented urinary incontinence. Statistical analysis shows no difference between the two groups, with a chi-square value of 1.49 ($p = 0.222$). Correlation between the size of the subjects and the onset of urinary incontinence produces a chi-square value equal to 16.81 ($p < 0.001$), showing a tendency of large subjects ($BW > 20$ kg) to aquire the disease. Concerning obesity, the possibile correlation between obese subjects before and after surgery and not-obese subjects ($\chi^2 = 3.647$; $p = 0.056$) is not significant, while the odds ratio value of 3.63 (1.13–11.64) appears meaningful. The therapeutic plan performed determined the complete disappearance of symptoms by the administration of a posology adapted for the specific requirements of each subject (Table I).

Blood analysis did not show any modification. To date, the longest duration of replacement oestrogenic therapy is 49 months.

TABLE I
Dosage and duration of oestrogenic replacement therapy in the nine bitches with after-spaying urinary incontinence

Subject	Maintenance dosage	Duration
Boxer	0.625 mg/12 h	9 months
Boxer	1.25 mg/96 h	6 months
Dobermann	0.625 mg/7 gg	37 months
German Shepherd	1.25 mg/24 h	25 months
German Shepherd	1.25 mg/48 h	49 months
German Shepherd	1.25 mg/24 h	49 months
Labrador Retriever	1.25 mg/24 h	5 months
Mixed breed	1.25 mg/72 h	19 months
Irish setter	1.25 mg/72 h	37 months

DISCUSSION

Incidence of after-spaying urinary incontinence incidence in our data was 5.1%, in agreement with Thrusfield *et al.* (1998), but less than the 20% reported by Arnold *et al.* (1989). The relationship between acquired urinary incontinence and the type of surgery performed did not show meaningful values, as also reported by Okkens *et al.* (1997), but in contrast with Osborne *et al.* (1999), who reported a possible greater frequency of urinary incontinence in ovariohysterectomized subjects. In agreement with literature (Gregory, 1994; Holt *et al.*, 1993), it is clear that large subjects (>20 kg), more than those of small or medium size, showed a greater predisposition to the disease. It was also found that bitches that were overweight before spaying had 3.5 times more risk of developing the disease as compared with subjects that were not obese before and after surgery, confirming that obesity could be a predisposing factor for urinary incontinence. Oestrogen replacement therapy as treatment for after-spaying urinary incontinence, once the exact posology was found for each subject, induced a complete regression of symptoms, without the evidence of oestrogen-related side effects.

REFERENCES

Arnold S. *et al.*, 1989. Incontinentia urinae bei der kastrierten hündin: häufigkeit und rassedisposition. *Schweiz. Arch. Tierheilk.*, **131**, 259–263

Creed, K.E., 1983. Effect of hormones on urethral sensitivity to phenylephrine in normal and incontinent dogs. *Research in Veterinary Science*, **34**, 177–181

Gregory, S.P., 1994. Developments in the understanding of the pathophysiology of the urethral sphincter mechanism incompetence in the bitch. *British Veterinary Journal*, **150**, 135–150

Holt, P.E. and Thrusfield, M.V., 1993. Association in bitches between breed, size, neutering and docking, and acquired urinary incontinence due to incompetence of the urethral sphincter mechanism. *The Veterinary Record*, **133**, 177–180

Miodrag, A. *et al.*, 1988. Sex hormones and the female urinary tract. *Drugs*, **36**, 491–504

Okkens, A.C. *et al.*, 1997. Comparison of long-term effects of ovariectomy versus ovariohysterectomy in bitches. *Journal of Reproduction and Fertility Supplement*, **51**, 227–231

Osborne, C.A. and Finco, D.R., 1999. *Nefrologia ed urologia del cane e del gatto* (Ed. UTET, Torino)

Thrusfield, M.V. *et al.*, 1998. Acquired urinary incontinence in bitches: its incidence and relationship to neutering practices. *Journal of Small Animal Practice*, **39**, 559–566.

Veterinary Research Communications, 28 (2004) 157–160

Signalling Compartmentalization Involved in the Boar Sperm Acrosome Reaction

B. Barboni, P. Lucidi*, N. Bernabò and M.G. Pistilli
Dipartimento di Scienze Biomediche Comparate, Facoltà di Medicina Veterinaria, Università degli Studi di Teramo, piazza Aldo Moro 45, 64100-Teramo, Italy
**Correspondence: Dipartimento di Scienze Biomediche Comparate, Facoltà di Medicina Veterinaria, Università degli Studi di Teramo, piazza Aldo Moro 45, 64100-Teramo, Italy*
E-mail: lucidi@unite.it

Keywords: boar, spermatozoa, capacitation, acrosome reaction, phospholipase C

Abbreviations: AR, acrosome reaction; CTC, chlortetracycline; PLC, phospholipase C; ZP, zona pellucida; cyto D, cytochalasin D; GaR, goat anti rabbit

INTRODUCTION

Actin, a major cytoskeletal protein involved in many functions in mammalian cells, supports the process of spermatogenesis by sustaining the morphological changes occurring in the germ cells as well as the acquisition of motility by the spermatozoon (Lecuyer *et al*., 2000). Moreover, the biological transformations that take place at a molecular level during the process of capacitation and leading to acrosome reaction also seem to be dependent on both actin localization and its polymeric status (F-actin). In the pig, as for other species, cytoskeletal reorganization has been related to either release of the acrosomal content or the fertilization rate (Castellani-Ceresa *et al*., 1993; Liu *et al*., 2002). It can be hypothesized that in the sperm the cytoskeleton acts as an organizer of specific domains on the plasma membrane. This arrangement occurs during the process of sperm maturation by interaction of the actin cytoskeleton with enzymatic or receptor proteins and/or fusogenic lipids in the membrane, allowing the translocation of specific molecules with antigenic properties to the sites where they are more directly involved in functional processes (AR, fusion etc.). On the basis of this knowledge, the authors have studied the process of capacitation in boar spermatozoa by modulating actin polymerization with cytochalasin D, a specific inhibitor of filament formation. In particular the effect of the actin cytoskeleton on a) acquisition of the capacitation pattern by CTC, and b) incidence of acrosomal exocytosis elicited in the sperm after exposure to zonae pellucidae proteins has been evaluated. Finally, the study has focussed on the major events taking place in the lag time from zona stimulation to acrosomal exocytosis, in particular i) the Ca waves triggered by the exposure of single sperm cells to solubilized ZP and ii) the localization of PLCγ_1, a membrane linked enzyme acting in the Ca-dependent transduction pathways of AR.

MATERIALS AND METHODS

The ejaculates of two boars of proven fertility were weekly recovered and immediately diluted in an extender type Modena to a final concentration of 2×10^7 sperm/mL, either with or without cyto D (20 μM). After at least 6 h of cyto D activity in the solutions, capacitation of each sample was carried out in a humidified incubator at 5% CO_2 at 38.5°C for 4 h, as previously described (Barboni *et al*., 1995). The semen samples were then submitted to the following procedures: the capacitation levels achieved by the sperm were recorded using CTC, which identifies a specific fluorescent pattern depending on the functional status of the cells (Mattioli *et al*., 1996). Briefly, the sperm were incubated for 30 s with CTC 750 μM on a warm stage, then fixed in glutaraldehyde (0.4% v/v) and inspected under a fluorescent microscope. The percentage of AR recorded in the sperm exposed to solubilized ZP was detected after treatment of the samples with 95% ethanol by using FITC-conjugated PSA, a specific agglutinin which reacts with the acrosomal content of intact cells. The Ca levels after exposure to ZP were evaluated using a confocal microscope equipped with an argon laser on single cells with a kinetic program. In brief, after coincubating the samples with the membrane permeant, Ca sensitive dye Fluo4-AM, a drop of washed spermatozoa was placed into a registration chamber coated with poly-L-lysine to allow adhesion of the cells. After a few scans, solubilized ZP (1ZP/μL) were added to the chamber and the Ca signalling recorded. The localization of PLCγ_1 was obtained by previously fixing (paraformaldeyde, 0.2%) and permeabilizing the cells (triton X-100, 0.1%); the sperm suspension was then incubated with anti PLCγ_1 (1:250) raised in rabbit and the secondary antibody GaR conjugated with Cy3.

RESULTS

The CTC staining carried out after four hours capacitation either with or without cyto D showed a similar proportion of cells that achieved the capacitated pattern (36.33 ± 6.03 vs 34.87 ± 2.2% respectively) characterized by a uniformly distributed fluorescence on the acrosomal region (Fig. 1). In spite of the attainment of this capacitated pattern, when the sperm were challenged with solubilized ZP, for samples capacitated under control conditions 42–6% of the cells underwent AR, but only 12–0.5% of cyto D-treated sperm were able to complete the exocytotic event (Fig. 2). The discrepancy of the obtained data suggests that actin polymerization is essential in order for the sperm to complete activation induced by the exposure to ZP with release of the acrosomal content. The measurement of intracellular Ca for single cells exposed to solubilized ZP did not show any significant differences in the kinetics of Ca signalling, or in the concentration achieved by sperm treated with cyto D, with respect to controls. In both groups, intracellular Ca levels peaked 20–30 s after ZP exposition, and gradually decreased to reach the basal concentration after 800 s. On the contrary, cyto D treatment influenced the localization of PLCγ_1, the enzyme

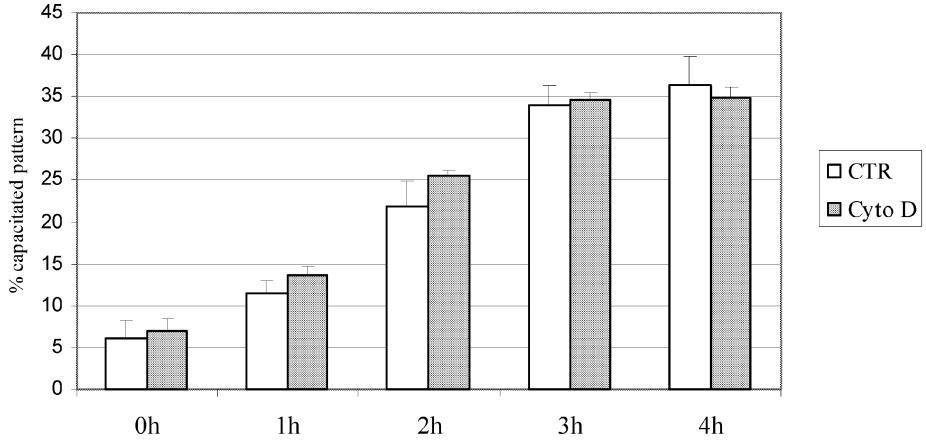


Figure 1. Incidence of the CTC capacitation pattern obtained for spermatozoa incubated *in vitro* either in the presence or the absence of cytochalasin D, an inhibitor of actin polymerization

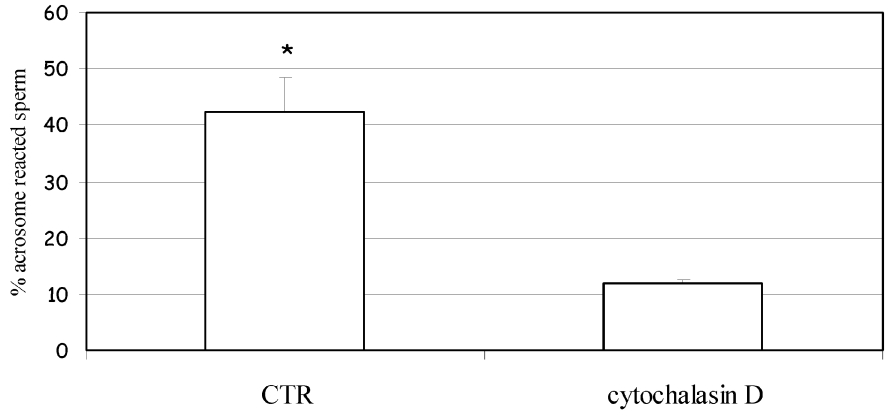


Figure 2. Percentage of AR sperm obtained by exposing semen capacitated *in vitro* for 4 h with or without cytochalasin D, to solubilized ZP

which contributes to Ca elevation after ZP stimulation: while in the control cells PLCγ_1 signal was mainly localized on the equatorial and postacrosomal regions with limited expression on the midpiece and tail, the sample treated with cyto D prevalently showed Cy3 positivity on the flagellum, together with a weak fluorescence at the postacrosomal level.

DISCUSSION

From the data obtained it can be assumed that actin polymerization is the sine qua non condition that allows the sperm to successfully perform the acrosome reaction after being engaged by ZP proteins. Nevertheless, by blocking actin filament formation, some modifications that take place during the process of capacitation can be prevented. Sperm treated with cyto D, even if they are able to gain the capacitated pattern with CTC, are not able to complete the process through the exocytosis of the acrosomal content after exposure to ZP. It can be supposed that the lack of PLCγ_1 expression at the equatorial level, as revealed by immunohistochemistry, could be responsible for this inability to undergo AR. This de-localization of the enzyme could cause an absent or limited release of intracellular Ca in specific subdomains, which may be pivotal for fusion of the plasma with the outer acrosomal membrane (Spungin *et al*., 1995). This was impossible to ascertain by evaluation of the whole calcium response of sperm as in our study. Experiments are in progress in order to verify both the changes of localized Ca fluxes in distinct regions of the sperm head and the co-localization of PLCγ_1 with actin filaments. These investigations could lead to a better understanding of the correlation between the actin polymerization status and the late events of the acrosome reaction, which take place after the engagement of sperm ZP receptors.

ACKNOWLEDGEMENTS

This work was supported by University funds (ex 60% 2003).

REFERENCES

Barboni, B., Mattioli, M. and Seren, E., 1995. Influence of progesterone on boar sperm capacitation. *Journal of Endocrinology*, **144**(1), 13–18

Castellani-Ceresa, L., Mattioli, M., Radaelli, G., Barboni, B. and Brivio, M.F., 1993. Actin polymerization in boar spermatozoa: fertilization is reduced with use of cytochalasin D. *Molecular Reproduction and Development*, **36**(2), 203–211

Lecuyer, C., Dacheux, J.L., Hermand, E., Mazeman, E., Rousseaux, J. and Rousseaux- Prevost, R., 2000. Actin-binding properties and colocalization with actin during spermiogenesis of mammalian sperm calicin. *Biology of Reproduction*, **63**, 1801–1810

Liu, D.Y., Martic, M., Clarke, G.N., Grkovic, I., Garrett, C., Dunlop, M.E. and Baker, H.W. 2002. An anti-actin monoclonal antibody inhibits the zona pellucida-induced acrosome reaction and hyperactivated motility of human sperm. *Molecular Human Reproduction*, **8**(1), 37–47

Mattioli, M., Barboni, B., Lucidi, P. and Seren, E., 1996. Identification of capacitation in boar spermatozoa by chlortetracycline staining. *Theriogenology*, **45**, 373–381

Spungin, B., Margalit, I. and Breitbart, H., 1995. Sperm exocytosis reconstructed in a cell-free system: evidence for the involvement of phospholipase C and actin filaments in membrane fusion. *Journal of Cell Science*, **108**, 2525–2535

Veterinary Research Communications, 28 (2004) 161–164

Ultrasonographic Study During Pregnancy of the Growth of an Encephalic Portion in the Canine Foetus

M. Beccaglia and G.C. Luvoni*
Department of Veterinary Clinical Sciences, Obstetrics and Gynaecology, University of Milan, Italy
**Correspondence: Department of Veterinary Clinical Sciences, Obstetrics and Gynaecology, University of Milan, Via Celoria, 10, 20133 – Milan, Italy*
E-mail: cecilia.luvoni@unimi.it

Keywords: dog, foetal brain, pregnancy, ultrasonography

Abbreviations: BP, biparietal; DPTV, deep portion of telencephalic vesicle; ICC, inner chorionic cavity

INTRODUCTION

Ultrasonographic identification and evaluation of foetal and extrafoetal structures allow determination of the gestational age and prediction of the delivery day for the bitch (Cartee and Rowles, 1984; England *et al*., 1990; Yeager *et al*., 1992; Gonzales-Bulnes *et al*., 1993; Moriyoshi *et al*., 1996; Luvoni and Grioni, 2000; Son *et al*., 2001). This represents a useful tool for canine reproductive programs, especially in the case of multiple or uncertain mating times. The research on new parameters to improve accuracy of the prediction of the day of parturition for the bitch prompted our previous investigation, which aimed at identifying encephalic foetal structures during pregnancy using ultrasonography. The deep portion of telencephalic vesicle (DPTV), represented by thalamus and basal nuclei primordia, was visualized between the 30th and 8th day before parturition as an ovoidal anechoic area with clearly defined margins (Beccaglia *et al*., 2003).

The aims of the present study were the ultrasonographic evaluation of DPTV growth during pregnancy in bitches of different sizes and the assessment of the suitability of DPTV measurement in comparison with measurement of the inner chorionic cavity diameter (ICC) and biparietal diameter (BP) which are usually adopted to predict the day of parturition for this species.

MATERIALS AND METHODS

For the present study serial ultrasonographic examinations were performed three times a week (from 18th–20th day after last mating to the day of parturition) for seven bitches, four Jack Russell terriers of 5–7 kg bodyweight (small size) and three mixed breed bitches of 18–25 kg bodyweight (medium size). The ultrasonographic examinations were made using a Medison SonoAce 8800 equipped with a 7.5 MHz convex

probe. Depending on the pregnancy period, ICC, or BP and DPTV were identified during each examination. The results were expressed as days before parturition (day 0). A linear regression model was adopted to analyse the relationship between the growth of DPTV and the days remaining to parturition. The equation obtained relating to the DPTV diameter was further applied to predict the day of parturition for bitches with unknown breeding dates. The results were compared to those obtained using ICC and BP measurements by the application of the equations described in a previous study (Luvoni and Grioni, 2000).

RESULTS AND DISCUSSION

The distribution of the data derived from DPTV measurement had a different trend for small (Fig. 1) and medium size (Fig. 2) dogs. For small dogs DPTV growth tended to reach a plateau, as shown from a significant ($p < 0.001$), but low regression coefficient ($R^2 = 0.70$). For medium size dogs linear growth was observed ($R^2 = 0.91$). The different first order coefficients denoted a different growth velocity of the DPTV in the two sizes of dog. The prediction of the delivery day derived from ICC and BP measurements, with an accuracy of ± 1 day, was significantly more reliable than that obtained using DPTV ($p < 0.05$), irrespective of bitch size (Table I). Extending the interval to ± 2 days, these differences were not observed for the small dog group and were smaller in the medium size dogs. Although both DPTV and BP were detected in the same gestational period, the low number of DPTV observations was due to its small diameter in the first period of pregnancy and to the almost complete calcification of the skull in the last week of pregnancy, both of which limit its measurement.

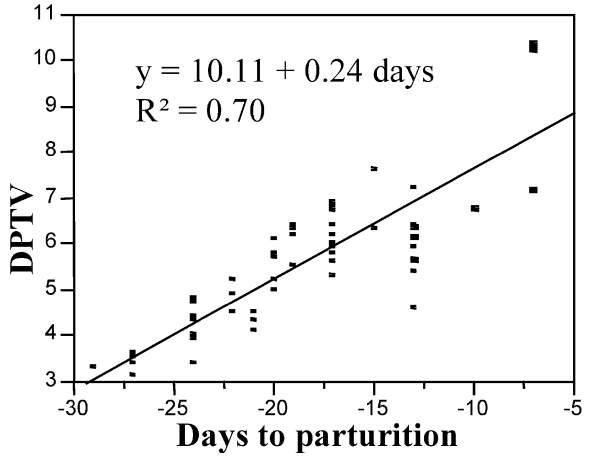


Figure 1. Relationship between DPTV diameter (mm) and days to parturition in small size bitches

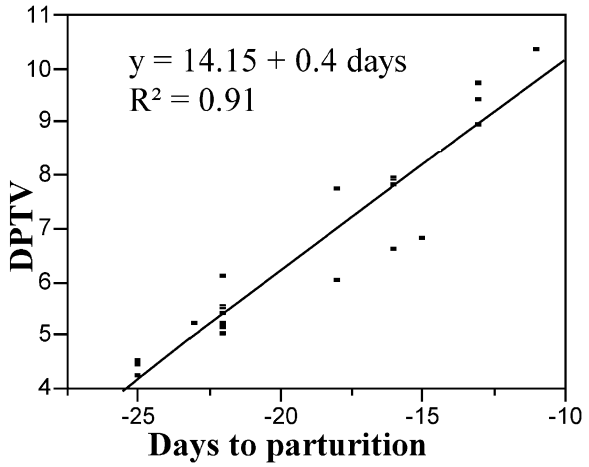


Figure 2. Relationship between DPTV diameter (mm) and days to parturition in medium size bitches

TABLE I
Prediction of delivery day, with an accuracy of ±1 day or ±2 days, obtained by the measurement of the diameter of the different extrafoetal and foetal structures

	Small size		Medium size	
	±1 day	±2 days	±1 day	±2 days
Inner chorionic cavity (ICC)	31/37 (83.8%)[a]	32/37 (86.5%)	17/20 (85%)[a]	18/20 (90%)[a]
Biparietal (BP)	46/62 (74.2%)[a]	54/62 (87.1%)	35/52 (67.3%)[a]	41/52 (78.8%)[ab]
Deep portion of telencephalic vesicle (DPTV)	4/10 (40%)[b]	8/10 (80%)	4/16 (25%)[b]	10/16 (62.5%)[b]

ab: different superscripts denote significant differences within columns ($p < 0.05$, Chi square test).

CONCLUSION

The results of this study showed that ultrasonographic evaluation of DPTV growth is possible for dogs of different sizes. This structure cannot be considered the optimal parameter to predict the day of parturition, but could be usefully combined with BP measurements in order to increase examination reliability.

REFERENCES

Beccaglia, M., Anfuso, F. and Luvoni, G.C., 2003. Ultrasonographic identification of fetal brain structures during pregnancy in the dog. In: J. Verstegen (ed), *Proceedings of the 3rd Annual Congress of the European Veterinary Society for Small Animal Reproduction*, Dublin-Ireland, 115–116

Cartee R.E. and Rowles, T., 1984. Preliminary study of the ultrasonographic diagnosis of pregnancy and fetal development in the dog. *American Journal of Veterinary Research*, **45**, 1259–1265

Gonzales-Bulnes, A., Herreros, M.A. and Botey, C., 1993. Estudio ecográfico de la gestatión en cánidos. *Medicina Veterinaria*, **10**, 345–354

England, G.C.W., Edward Allen, W. and Porter, D.J., 1990. Studies on canine pregnancy using B-mode ultrasound: Development of the conceptus and determination of gestational age. *Journal of Small Animal Practice*, **31**, 324–329

Luvoni G.C. and Grioni, A., 2000. Determination of gestational age in medium and small size bitches using ultrasonographic fetal measurements. *Journal of Small Animal Practice*, **41**, 292–294

Moriyoshi, M., Waki, Y., Nakao, T. and Katawa, K., 1996. Observation of the growth process of a beagle embryo and fetus by ultrasound. *Journal of Veterinary Medical Science*, **58**, 443–445

Son, C.H., Jeong, K.A., Kim, J.H., Park, I.C., Kim, S.H. and Lee, C.S., 2001. Establishment of the prediction table of parturition day with ultrasonography in small pet dogs. *Journal of Veterinary Medical Science*, **63**, 715–721

Yeager, A.E., Mohammed, H.O., Meyers-Wallen, V., Vannerson, L. and Concannon, P.W., 1992. Ultrasonographic appearance of the uterus, placenta, fetus, and fetal membranes throughout accurately timed pregnancy in Beagles. *American Journal of Veterinary Research*, **53**, 342–351

Veterinary Research Communications, 28 (2004) 165–168

The Correlation Between Mast Cells and Some Inflammatory Mediators in the Bovine Endometrium

S. Belluzzi[1]*, M. Galeotti[2], C.M. Eutizi[3] and C. Castagnetti[1]
[1] *Veterinary Clinical Department – Obstetrical and Gynaecological Section – Faculty of Veterinary Medicine, University of Bologna, Via Tolara di Sopra 50, 40064, Ozzano Emilia, Bologna, Italy;* [2] *Animal Production Sciences Department – Faculty of Veterinary Medicine, University of Udine, Via delle Scienze 208, Udine;* [3] *Veterinary Practitioner*
* *Correspondence: Prof. Stefano Belluzzi, Dipartimento Clinico Veterinario, Sezione Ostetrico-Ginecologica, Via Tolara di Sopra 50, 40064, Ozzano Emilia, Bologna, Italy*
E-mail: belluzzi@vet.unibo.it

Keywords: cow, mast cells, leukotriene, endometrium

Abbreviations: LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; IL-6, interleukin-6; TXB_2, thromboxane B_2

INTRODUCTION

The endometrial cellular infiltrate has been the subject of various studies aimed at determining and clarifying its physiological and pathological importance. Gonzales *et al.* (1985) classified the endometria of non-pregnant cows into four categories and showed how, as the grade of severity of endometrial damage increases, the number of mast cells, which are considered fundamental in beginning and propagating the inflammatory process, increased notably. The correlation between endometrial mast cells and the quantity of some inflammatory mediators was studied by Belluzzi *et al.* (1994) who found a close correlation between the elevated values of leukotriene B_4 (LTB_4) and the granulocytic tissue infiltrate. No correlation was found for mast cells. The difference in the average values of LTB_4 and thromboxane B_2 (TXB_2) found in the endometria of cows having clinically evident endometritis and in healthy cows having normal reproductive activity was statistically significant for both mediators. A precise evaluation of the distribution of mast cells in the endometria of cows was carried out by Galeotti *et al.* (1997) on the uteri of prepubertal and pubertal bovines, with the aim of establishing normal reference values. Other interesting studies on endometrial distribution and on the typifying of mast cells were carried out by Welle *et al.* (1997) on horses and by Küther *et al.* (1998) on bovines. These last studies classified the mast cells present in various organs and in the uterus into three sub-groups on the basis of their neutral protease content; thus mast cells containing tryptase, chymase or both were identified. The aims of this study were to evaluate the distribution and the number of mast cells present in the bovine endometrium and to compare them with the reference values (Galeotti *et al.*, 1997) to verify their eventual

correlation with endometrial concentrations of leukotriene B_4, leukotriene C_4 and interleukin-6 (IL-6), all of which are inflammatory mediators potentially released by mast cells.

MATERIALS AND METHODS

12 Holstein-Friesian cows between three and eight years of age culled for various reasons were studied. 24 h before slaughtering a bioptic endometrial sample and a blood sample were taken. The bioptic samples were dosed with LTB_4 and LTC_4 after column purification and with IL-6 without purification employing an EIA kit which uses human monoclonal antibodies. After slaughtering, five samples were taken from each uterus: one from the body, one from the base of each horn and one from the apex of each horn. The samples were fixed in buffered formalin and were then processed using normal histological techniques and stained with hematoxylin-eosin and toluidine blue in order to highlight the mast cells. The count of these mast cells was carried out in five optic fields (400 ×) for each of the five sample sites, according to a method described by Galeotti *et al.* (1997). The hematic progesterone was dosed in order to evaluate the phase of the estral cycle. The statistical comparison between the values of the mediators and the number of mast cells counted at the base of the horn was carried out using linear regression. The comparison between the values of the mediators and mast cells in healthy endometria with respect to pathological ones was carried out using the non-parametric test according to Wald-Walfowitz. The values of the mast cell infiltrate in the five sample sites were compared with the non-parametric test for dependent samples according to Wilcoxon.

RESULTS AND DISCUSSION

The histological evaluation of the degree of total cellular infiltrate (granulocytes, lymphocytes, plasmacytes) allowed us to identify four uteri affected by endometritis and eight healthy ones. The dosage of IL-6 furnished values all below the calibration curve (5 pg) and were therefore not considered valid. The average values of LTB_4 and LTC_4, taken from the bioptic sample, and the average value of the mast cells at the base of the horn and in the five samples taken post-mortem from healthy cows and those with endometritis are reported in Table I.

25% of the cows examined had different histological reports in the different sites of evaluation. This result could be a cause of disagreement regarding the representativeness of a single biopsy. The count of mast cells carried out using toluidine blue stain permitted us to confirm that the values found in the endometria deemed healthy are in agreement with the normal value defined by Galeotti *et al.* (1997). The differences between the average values of mast cells present in healthy endometria with respect to the pathological ones are not significant however, nor are the differences in average

TABLE I
Average values of LTB_4 and LTC_4 found in the bioptic samples of healthy bovines and those with endometritis and average values of the mast cells found in the five samples taken postmortem from healthy bovines and those with endometritis

	Biopsy *in vivo*				Post mortem Mast cells	
	LTB_4 pg/mg tissue	LTB_4 pg/mg protein	LTC_4 pg/mg tissue	LTC_4 pg/mg protein	Base of the horn	General
Normal endometria	86.35	7.32	29.51	1.39	7.13	7.75
Pathological endometria	72.34	3.88	45.82	2.62	9.15	11.80

concentrations of the two mediators in the two categories. However, a slight increase of LTC_4 *in uteri* with endometritis was observed, a category where the average count of mast cells was higher. The distribution of mast cells in the uterus does not seem to have distinctive characteristics in the five sites examined, since only the differences between the average values of the mast cells in the body with respect to the apex of the right horn were significant. This result is difficult to interpret and could be correlated with the number of pregnancies sustained by the horn and/or the site of the most recent pregnancy. Our research did not show any significant relationship between the number of mast cells present at the base of the horn and the value of the eicosanoids LTB_4 and LTC_4 obtained from bioptic sample at the same site. We need to consider that the staining using toluidine blue carried out by us shows only non-degranulated mast cells and therefore does not give an indication of the relationship between degranulated and non-degranulated mast cells in the optic field under examination. Other staining techniques (Küther *et al*., 1998) showed mast cells which do not coincide with those shown by toluidine blue in bovine endometria populations and, therefore, the technique used to visualize the mast cells can condition the result. A second consideration regards the mediators investigated in this study, that is LTB_4, LTC_4 and IL-6, which are indicated in the literature as also being released by mast cells. However, at this time, there is no study that demonstrates their release in animal endometria. On the other hand, the relationship between the LTB_4 values and the neutrophil leukocyte infiltrate seems to be verified (Belluzzi *et al*., 1994). The test results of the dosage of IL-6 suggest that the human monoclonal kit does not have a cross-reaction with bovine endometrial tissue. In conclusion, this study did not show any strict correlation between the inflammatory mediators examined and the mast cells highlighted with toluidine blue. Histochemical and immunohistochemical staining could furnish more reliable data on the number of mast cells present in the endometrium. The possibility that the mediators investigated and correlated with the

mast cells are also released by other cells constituting the endometrial infiltrate suggests that we turn our attention to substances which are more specifically attributable to mast cells, such as histamine, tryptase and chymase.

ACKNOWLEDGEMENTS

The LTB_4, LTC_4 and IL-6 determinations were performed by Dr. Stefano Boschi, Service of Clinical Pharmacology, S. Orsola-Malpighi Hospital, University of Bologna.

REFERENCES

Belluzzi, S., Galeotti, M., Carluccio, A., Matteuzzi, A. and Boschi, S., 1994. Raffronto tra parametri istologici e livelli di mediatori chimici della flogosi in biopsie endometriali di bovine ipofertili. *Proc. XVIII World Buiatrics Congress*, Bologna, **1**, 313–316

Galeotti, M., Belluzzi, S., Volpatti, D., Bergonzoni, M.L., D'Agaro, E. and Volpelli, L.A., 1997. Evaluation of mast cells in calf and heifer uteri. *Theriogenology*, **48**, 1301–1311

Gonzales, H.E., Crowell, W.A., Caudle, A.B. and Thompson, F.N., 1985. Morphometric studies of the bovine uterus: microscopic lesion and retrospective reproductive history. *American Journal of Veterinary Research*, **46**, 2588–2595

Küther, K., Audigé, L., Kube, P. and Welle, M., 1998. Bovine mast cells: distribution, density, heterogeneity, and influence of fixation techniques. *Cell Tissue Research*, **293**, 111–119

Welle, M.M., Audigé, L. and Belz, J.P., 1997. The equine endometrial mast cell during the puerperal period: evaluation of mast cell number and types in comparison to other inflammatory changes. *Veterinary Pathology*, **34**, 23–30

Veterinary Research Communications, 28 (2004) 169–172

Clinical Use of Twice Daily Injections of Buserelin Acetate to Induce Ovulation in the Mare

F. Camillo, M. Pacini, D. Panzani, I. Vannozzi, Al. Rota* and G. Aria
Department of Veterinary Clinic, Università degli Studi di Pisa, Italy
**Correspondence: Dipartimento di Clinica Veterinaria dell'Università degli Studi di Pisa, Via Livornese (lato monte), 56010 S. Piero a Grado, Pisa, Italy*
E-mail: fcamillo@vet.unipi

Keywords: mare, buserelin acetate, induction of ovulation

Abbreviations: hCG, human chorionic gonadotrophin; CEG, crude equine gonadotrophin; GnRH, gonadotrophin-releasing hormone

INTRODUCTION

The difficulties in the prediction of ovulation time in the mare, together with the increasing use of cooled and frozen semen for artificial insemination, have stimulated investigators to search for methods for induction of ovulation. These methods are essentially based on exogenous administration of one of three hormones directly or indirectly involved in the mechanism of ovulation: hCG (Human Chorionic Gonadotrophin), CEG (Crude Equine Gonadotrophin) (Duchamp *et al*., 1987), or GnRH (Gonadotrophin-releasing hormone). Repeated administration of hCG, which is a heterologous glycoprotein for mares, may stimulate an immune response able to neutralise the effect of the hormone (Roser *et al*., 1979). Although CEG has not shown this negative side effect, it is not found on the market, and purified extracts are produced and utilised only by few research groups. GnRH, physiologically released in a pulsatile manner, and its analogues have been tested for induction of ovulation in oestrous mares either by repeated injections at given time intervals (Palmer and Quellier, 1988) or as slow-release subcutaneous implants. For this purpose, a slow-release subcutaneous implant containing the synthetic GnRH analogue deslorelin acetate (Ovuplant®) is successfully used in United States and Australia (Meinert *et al*., 1993; Meyers *et al*., 1997). The response to Ovuplant® administration was similar to that obtained using hCG. However, this formulation is currently not available in the Italian and European market. The efficacy of buserelin, a GnRH analogue also commercialised in Italy, has been recently investigated for induction of ovulation in the mare. The administration of a single dose has not yielded encouraging results (Vidament *et al*., 1992). On the other hand, Battut *et al*. (2001) observed that most mares treated twice daily with intravenous (IV) administration of 20 or 40 μg of buserelin ovulated within 48 h. The aim of our study was to evaluate the clinical efficacy of repeated administration of 40 μg buserelin for induction of ovulation in the mare, as compared to a single administration of 2500 IU hCG or a placebo.

MATERIALS AND METHODS

During three breeding seasons, 37 Haflinger mares aged 2–5 years were kept in open paddocks and fed hay ad libitum. Their ovarian activity was monitored daily by transrectal ultrasonography (Dynamic Imaging System 2000, and a 5 MHz linear probe) for 83 oestrous cycles. When a dominant follicle equal to or larger than 32 mm in diameter was detected, together with a uterus showing the typical signs of oestrus, ultrasonographic examinations were intensified to one every 12 h, and mares were assigned to one of the three following treatments: Group C (control), treated with a placebo, consisting of 2 ml distilled water IV, twice daily until ovulation ($n = 19$); Group Bu40, treated with buserelin acetate, 40 μg IV, twice daily until ovulation ($n = 22$); and Group hCG, treated with a single IV administration of 2500 IU hCG ($n = 42$). Mares were artificially inseminated in 29 cycles every 48 h from the day following the onset of treatment until ovulation, using at least 500×10^6 motile spermatozoa obtained from a stallion of proven fertility. At day 7 post-ovulation, 20/29 mares were subjected to uterine flushing for embryo recovery, while for the remaining 9 mares pregnancy diagnoses were carried out ultrasonographically at 14 days post-ovulation. Data are shown as means – standard deviation, and were analysed using the Student's *t*-test and Chi-squared-test.

RESULTS

Over 83 oestrous cycles, 86 ovulations were observed: 78 single and 4 double ovulations, and one luteinization. The number of ovulations per cycle and the fertility of treated mares were not influenced by treatment. The mean time from onset of treatment to ovulation was significantly anticipated for both Group Bu40 and Group hCG, as compared to the control, and ovulation occurred earlier for mares belonging to Group hCG. Likewise, the proportion of ovulations occurring within 48 h of treatment was significantly lower for the control group, as compared to treated mares, while of those occurring between 36 and 48 h after treatment the proportion was 88.1% for Group hCG, significantly higher than for Group Bu40 (22.7%) and Group C (5.3%), which had no significant differences between them. Results are shown in detail in Table I.

DISCUSSION

The results of this study showed that, although the buserelin protocol hastened ovulations in mares without interfering with their fertility as compared to the control group, its efficacy was clearly lower than that of the hCG protocol. These results are only partially in agreement with those found in the literature: Squires *et al.* (1988)

TABLE I
Responses to the administration of a placebo, twice daily (Group C), 40 μg buserelin, twice daily (Group Bu40), or 2500 IU hCG, once (Group hCG)

	Group C	Group Bu40	Group hCG
Ovulations/cycle	20/19	23/22	43/42
Interval treatment-ovulation	113.1 ± 49.7^{A}	$74.9 \pm 41.9^{B,a}$	$46.7 \pm 7.4^{D,c}$
Ovulations within 48 h	$5.3\%^{A}$	$42.8\%^{C,a}$	$97.6\%^{D,d}$
Ovulations between 36 and 48 h	$5.3\%^{A}$	$22.7\%^{A}$	$88.1\%^{D}$
Diameter of preovulatory follicle	44.9 ± 7.1^{A}	$40.9 \pm 5.9^{A,a}$	$38.1 \pm 3.9^{D,a}$
Pregnancies/cycle	10/15	7/10	4/4

Within a line, different subscripts indicate significant differences:
$A \neq B$ or $a \neq b = p < 0.05$; $A \neq C$ or $a \neq c = p < 0.01$; $A \neq D$ or $a \neq d = p < 0.001$.

showed a mean time from the beginning of twice daily administration of 40 μg buserelin and ovulation of 45.6 ± 15.2 h, much shorter than the 74.9 ± 41.9 h found in our study. Similarly, Battut *et al.* (2001), when comparing the twice daily administration of 40 and 20 μg buserelin, reported a higher proportion of ovulations within 48 h of the start of the treatment (83% and 90% respectively), as compared to that observed in our study (43%). The aim, when inducing ovulation in mares, is not so much to hasten it, but to ensure that it occurs in a short and predictable time interval, in order to plan when the mare should be artificially inseminated. Contrary to what has been described in the literature, in our study treatment with buserelin did not seem to fulfil these criteria, as it was not able to induce ovulation in the mare in a repeatable and predictable way.

REFERENCES

Battut, B., Lepoutre, N., Trocherie, E., Hect, S., Grand Champ Des Raux, A., Nicaise, J.L., Verin, X., Bertrand, J., Fieri, F., Hoier, R., Renault, A., Egron, L., Tainturier, D. and Bruyas, J.F., 2001. Use of buserelin to induce ovulation in cyclic mares. *Theriogenology*, **55**, 1679–1695

Duchamp, G., Bour, B., Combarnous, Y. and Palmer, E., 1987. Alternative solutions to hCG induction of ovulation in the mare. *Journal of Reproduction and Fertility*, Supplement **35**, 221–228

Meinert, C., Silva, J.F., Kroetz, I., Klug, E., Trigg, T.E., Hoppen, H.O. and Jochle, W., 1993. Advancing the time of ovulation in the mare with a short-term implant releasing the GnRH analogue deslorelin. *Equine Veterinary Journal*, **25**, 65–80

Meyers, P.J., Bowman, T., Blodgett, G., Conboy, H.S., Gimenez, T., Reid, M.P., Taylor, B.C., Thayler, J., Jochle, W. and Trigg, T.E., 1997. Use of the GnRH analogue desloreline acetate in a slow-release implant to accelerate ovulation in oestrus mares. *Veterinary Record*, **140**, 249–252

Palmer, E. and Quellier, P., 1988. Uses of LHRH and analogues in the mare. *Proceedings of the 11th International Congress Animal Reproduction and Artificial Insemination*, Dublin, **5**, 339–346

Roser, J.F., Kiefer, B.L., Evans, J.W., Neely, D.P. and Pacheco, C.A., 1979. The development of antibodies to human chorionic gonadotrophin following its repeated injection in the cyclic mare. *Journal of Reproduction and Fertility*, Supplement **27**, 173–179

Squires, E.L., Harrisson, L.A., Mckinnon, A.O. and Voss, J.L., 1988. Use of hCG, GnRH agonist or

prostaglandin analogue for induction of ovulation in mares. *Proceedings of the 11th International Congress Animal Reproduction and Artificial Insemination*, Dublin, **5**, 460–462

Vidament, M., Arnaud, G., Trillaud-Geyl, C., Duchamp, G. and Palmer, E., 1992. Analogue of GnRH (buserelin) and of PGF2a do not induce ovulation in mares. *Proceedings of the 12th International Congress Animal Reproduction and Artificial Insemination*, The Hague, **4**, 1927–1929

Veterinary Research Communications, 28 (2004) 173–176

Effect of Cryoprotectant Agents on the Potential Development of Sheep Preantral Follicles

G. Capacchietti[1], S. Cecconi[2], L. Gioia[1] and M. Turriani[1]
[1] *Dip. Scienze Biomediche Comparate, Università degli Studi di Teramo;*
[2] *Dip. Scienze e Tecnologie Biomediche, Università degli Studi di L'Aquila*
* *Correspondence: Dip. Scienze Biomediche Comparate – sezione Fisiologia – Facoltà di Medicina Veterinaria, Università degli Studi di Teramo, Piazza Aldo Moro, 45, 64100 Teramo, Italy*
E-mail: segifv@ifv.vet.unite.it

Keywords: sheep, ovary, cryoprotector agents, preantral follicle, oocyte

INTRODUCTION

The storage of ovarian cortical strips has been proposed as a strategy for preservation of fertility, especially for young cancer patients or for women at risk of premature ovarian failure. In fact, the possibility of restoring fertility and achieving pregnancy after transplantation of cryopreserved ovarian cortical strips has been reported for large mammals (Gosden *et al*., 1994), rodents (Cox *et al*., 1996) and recently for humans (Oktay, 2001). In effect, primary/secondary follicles survive cryopreservation and grafting procedures in a large number, and can undergo subsequent development. However, the applicability of this procedure is affected by two main problems: firstly, the chemical and physical damage induced by the cryopreservation protocol, which results in a dramatic loss of potentially developing follicles; secondly, reimplantation of a stored ovarian cortex after chemotherapy does not exclude an eventual cancer relapse. Thus, an alternative option to preserve fertility relies on the possibility of obtaining mature oocytes from in vitro cultured small follicles isolated from frozen/thawed tissue. Also in this case it is necessary to develop cryopreservation protocols able to preserve follicle functionality and to identify culture conditions capable of successfully supporting full follicle development. The aim of our study was to compare the in vitro development of intact sheep preantral follicles isolated from cryopreserved ovarian cortical strips stored with two widely used CPAs, DMSO and EG.

MATERIALS AND METHODS

Prepubertal sheep ovaries were collected from the local abattoir and transported to the laboratory in a thermostatic container at 30°C within 1 h of collection. The ovaries were cut with surgical blades into fragments of about 0.5×0.5 cm that were

used to isolate unfrozen preantral follicles or to cryopreserve ovarian strips. Dimethyl sulfoxide (DMSO) or ethylene glycol (EG) were used at the final concentration of 1.5 M in M2 medium supplemented with 10% FCS, antibiotics (75 mg/l penicillin-G, 50 mg/l streptomycin sulfate) and 0.1 M sucrose. Ovarian pieces were firstly aspirated into plastic insemination straws (IMV, France), kept on ice for 15 min and transferred to a programmable freezer (Cryo-Logic, IVM, France). The straws were cryopreserved according to a slow cooling protocol developed by Gosden *et al.* (1994) then plunged into liquid nitrogen and stored for up to 3 months. Thawing was carried out by rapidly warming the straws to 38°C. Dilution of the cryoprotectants was performed at room temperature with HEPES-buffered TCM199 supplemented with decreasing concentrations of DMSO or EG and 0.1 M sucrose (3 steps, 15 min). Preantral follicles were mechanically isolated from either frozen or fresh ovary fragments in Hepes-buffered TCM199. Preantral follicles were then measured, and selected follicles of a mean diameter of 170 ± 20 μm were individually transferred to 96-V-well microtiter plates and cultured in 25 μl of α-MEM supplemented with 2% FCS, 1% ITS, antibiotics and 1 μg/ml ovine FSH (oFSH). Culture was carried out for 10 days at 38°C in a O_2 5%, CO_2 5%, N_2 90% gas mixture (Cecconi *et al.*, 1999). Culture media were changed daily and 15 μl of recovered conditioned media was stored at –80°C for estradiol determination. At the end of culture follicles were analysed to record: 1) diameter; 2) percentage of differentiated antral-like cavities; 3) morphological aspect (by histological analysis) and 4) estradiol production. Cumulus oocyte complexes (COCs) were isolated from in vitro growth antral follicles and analysed to record: 1) percentage of isolated healthy oocytes presenting a surrounding layer of cumulus cells and 2) the metabolic coupling between germinal and somatic compartment (by evaluation of the ^{3}H-uridine uptake).

All parameters were evaluated in parallel for early antral follicles isolated from fresh ovaries.

RESULTS

The first series of experiments was performed in order to compare the morphological architecture of preantral follicles isolated from fresh or frozen ovarian cortex. The majority (94%; $n = 33$) of preantral follicles isolated from the fresh cortex showed the presence of oocytes with agranular cytoplasm and a central GV, surrounded by several layers of granulosa cells and a well organized theca layer. In contrast, 50% of EG ($n = 15$) and 34% of DMSO ($n = 15$; EG vs. DMSO: $p < 0.01$) cryopreserved follicles revealed a damaged structure, frequently characterized by loss of intercellular communication between the oocyte and the surrounding granulosa cells, and signs of pyknosis in the GV as well as in some granulosa cells. Regarding growth, freshly isolated and frozen preantral follicles grew from a mean diameter of about 170 μm to a final diameter of approximately 300–310 μm after 10 days of culture, without

TABLE I
In vitro development of sheep preantral follicles obtained from frozen or fresh ovarian cortex and cultured for 10 days

Treatment	No. of follicles	Follicle diameter (□m)				Antral like cavity formation (%)	Healthy COCs (%)
		Days of culture				Days of culture	
		0	2	6	10	10	
Fresh	160	172±4	230±4	258±4	310±8	78±3	56±3
EG	159	183±3	232±5	247±3	307±4	73±3	42±5*
DMSO	176	177±5	229±3	250±4	300±3	70±3	25±4**

significant differences between the experimental groups tested. A translucent antral-like cavity was recognizable starting from day 6 and, by the end of culture, the percentage of follicles forming antral-like cavities was similar for fresh and frozen follicles. This result was confirmed by histological analysis, which evidenced a similar morphological aspect between IVG antral follicles derived from fresh or cryopreserved tissue.

Fresh follicles released increasing amounts of E_2 with a sharp rise from day 6 to day 10 ($p < 0.01$). A similar final level of this steroid was found for EG cryopreserved follicles, although lower than for the group control, up to day 6 ($p < 0.05$). E_2 production by DMSO treated follicles was comparable to that of those treated with EG up to day 6, but from that day onwards it remained significantly lower as compared with fresh and EG groups ($p < 0.01$).

On day 10, COCs were recovered from IVG antral follicles and their morphological aspect was assessed. As shown in Table I, the percentage of healthy COCs derived from IVG unfrozen follicles was significantly higher as compared to that of cryopreserved follicles, even though EG appeared to more efficiently protect COC quality. The extent of metabolic co-operation between the somatic and germinal compartments was measured by comparing coupling index (CI) values between the different experimental groups. CI values were loosely sorted into two classes, termed "high" (range 10–30) and "low" (<10). 100% and 80±10% of COCs obtained from control and IVG fresh follicles, respectively, displayed high CI values in comparison with 51±6% of the EG and 35±7% of the DMSO group.

DISCUSSION

In this study we demonstrated that cryopreservation of sheep cortical tissue allows the recovery of viable preantral follicles, which can be grown in vitro with differing

efficiency depending on the cryoprotective agent employed. Our results confirm previous observations indicating that the freezing/thawing process severely damages preantral follicles, which are considered less resistant than primordial ones to the injuries caused by cryopreservation (Gosden *et al*., 2002). The increased complexity of the growing follicles makes them more sensitive to cryodamage. It is well known that the physiological differentiation of ovarian follicles in mammalia is strictly dependent on the existence of a bi-directional regulative dialogue between the germinal and somatic compartments, exerted through the production of paracrine/autocrine factors, or through the presence of functional gap junctions (Cecconi, 2002). From our results, evaluation of the post-thaw viability of the follicles using morphological analysis shows that cryopreservation can cause disruption of gap junctional communication, thus impairing follicle survival. The extent of such damage is dependent on the cryoprotectant used, as demonstrated by the finding that the percentage of healthy follicles is higher in EG than DMSO. Our results also demonstrated that the in vitro culture of sheep preantral follicles is a useful test for evaluating the damage induced by CPA and/or freezing procedures, as previously proposed for the mouse (Newton *et al*., 2001) and the sheep (Amorin *et al*., 2003). However, a comprehensive evaluation of cryodamage cannot be exclusively based upon morphological analysis but also needs to be assessed by more detailed biochemical investigations.

ACKNOWLEDGEMENTS

This work was supported by Università degli Studi di Teramo, Esercizio Finanziario 40% 2000.

REFERENCES

Amorim, C.A., Rondina, D., Rodrigues, A.P.R., Costa, S.H.F., Goncalves, P.B.D., de Figueiredo, J.R. and Giorgetti, A., 2003. Isolated ovine primordial follicles cryopreserved in different concentrations of ethylene glycol. *Theriogenology*, **60**, 735–742

Cecconi, S., 2002. Growth and differentiation of small ovarian follicles in mammals: problems and perspective. *Journal of Reproduction Development*, **48**, 431–445

Cecconi, S., Barboni, B., Coccia, M. and Mattioli, M., 1999. In vitro development of sheep preantral follicles. *Biology of Reproduction*, **60**, 594–601

Cox, S.L., Shaw, J. and Jenkin, G.J., 1996. Transplantation of cryopreserved fetal ovarian tissue to adult recipients in mice. *Journal of Reproduction and Fertility*, **107**, 315–322

Gosden, R.G., Baird, D.T., Wade, J.C. and Webb, R., 1994. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at −196 degrees. *Human Reproduction*, **9**, 597–603

Gosden, R.G., Mullan, J., Picton, H.M., Yin, H. and Tan, S.L., 2002. Current perspective on primordial follicle cryopreservation and culture for reproductive medicine. *Human Reproduction Update*, **8**, 105–110

Newton, H. and Illingworth, P., 2001. *In-vitro* growth of murine pre-antral follicles after isolation from cryopreserved ovarian tissue. *Human Reproduction*, **16**, 423–429

Oktay, K., 2001. Ovarian tissue cryopreservation and transplantation: preliminary findings and implications for cancer patients. *Human Reproduction Update*, **7**, 526–534

Veterinary Research Communications, 28 (2004) 177–179

Quantitative Motor Unit Action Potential Analysis in Skeletal Muscles in Horses and Ponies

B. Ciminaghi, M. Albertini*, M. Costanzi, F. Patrese and M.G. Clement
Department of Animal Pathology, Hygiene and Public Veterinary Health – Section of Biochemistry and Physiology, University of Milan, Via Celoria, 10, 20133 Milan, Italy
**Correspondence: Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Biochimica e Fisiologia, Facoltà di Medicina Veterinaria, Via Celoria,10, 20133 Milano, Italy*
E-mail: Mariangela.albertini@unimi.it

Keywords: electromyography, horse, pony

Abbreviations: EMG, electromyography; MUP, motor unit potential

INTRODUCTION

Electromyography (EMG) is a non-invasive technique for evaluating the electrical activity of the motor units (MUPs) (Platt, 2002). The first electromyographic studies were performed by Adrian and Bronk in 1929, by means of a specially manufactured electrode called concentric needle. In this method the electromyographers assess the traces empirically, by viewing them on an oscilloscope screen and by listening to their sound on an audio monitor, providing a semiquantitative evaluation of the muscle activity (Adrian and Bronk, 1929). Recently, Wijnberg *et al.* (2002) performed, in horses, an offline analysis of the MUPs in a semiautomatic way. This method, where the MUPs were not automatically selected and were manually analyzed, is quite reliable and easy to use. However, to observe the results in real time, we used a multi-MUP EMG analysis, based on a "turns" analysis described by Willison (1964) and refined by Stalberg *et al.* (1995). The main advantages of this method are that it is an online method, which permits an automatic expression of more than one MUP, it is fast enough for routine use, it does not require special electrodes and it is reproducible. The aim of our study was to create a normative database that can be used for electromyographic evaluation of muscular function in clinical studies and to compare the MUP parameters between the two groups of animals which are characterized by a different muscular mass.

MATERIALS AND METHODS

Electromyographic examination was performed in 10 healthy and not sedated horses, 4 male and 6 female, and in 9 healthy and not sedated ponies, 3 male and 6 female,

using portable commercial EMG equipment (Keypoint, Dantec) with dedicated software. The animals were grounded using a subcutaneous needle electrode (ground electrode), positioned near a bony prominence. The EMG examination was performed by inserting a disposable concentric needle electrode into the muscle, advancing or withdrawing it in small steps and maintaining it in each new position with a firm grip, to sample different areas of the muscle. The EMG examination was performed in splenius, trapezius, and triceps muscles. Each muscle was evaluated at rest and during different degrees of muscle contraction. The software proposes a grouping of the MUPs, and many different MUPs were usually obtained from each recording site. First of all, the MUPs were divided into monophasic and polyphasic, according to the number of phases, and thus, for each subset of MUPs, the program analysed duration, amplitude, area/amplitude ratio and firing rate. All values are expressed as mean – standard deviation (s.d.). The statistical significance of differences between the two sets of data was assessed using the two tailed t-test for unpaired data. A p value < 0.05 was regarded as a statistically significant difference.

RESULTS

The mean values of the main parameters evaluated in splenius, trapezius and triceps muscles of the two groups of animals are reported in Table I. Our data shows that the number of MUPs is significantly greater for ponies, showing that it is easier to perform the EMG analysis for ponies. Therefore, our results demonstrate that the percentage of the polyphasic MUPs and the area/amplitude ratio are significantly lower for ponies, while the duration and the firing rate are significantly higher than those for horses. In contrast, no difference between the two groups was detected for the amplitude logarithm.

DISCUSSION

This study presents normative data for healthy animals, which can be used for electromyographic evaluation of muscular function in clinical studies. Our results show that the parameters characterizing the MUPs in the two groups of healthy animals are significantly different, indicating that EMG can be influenced by different muscular mass and by the different composition of motor units.

ACKNOWLEDGEMENTS

This work was supported by FIRST 2002 and by COFIN 2002.

REFERENCES

Adrian, E.D. and Bronk, D.V., 1929. Discharge of impulses in motor nerve fibres. The frequency of discharge in reflex and voluntary contraction. *Journal of Physiology*, **67**, 119–151

TABLE I
Main electromyographic parameters evaluated in 10 horses and in 9 ponies

Horse	n. MUP	% Poli.	Log ampl.	Dur.	Ar/Ampl	Fr
1	73	6.6	2.57	9.56	1.67	5.12
2	80	5.0	2.54	7.60	1.32	4.26
3	45	10.0	2.57	8.26	1.55	4.01
4	45	4.0	2.52	7.96	1.56	4.02
5	71	7.0	2.58	7.23	1.04	12.99
6	56	8.0	2.54	7.51	1.29	4.84
7	57	14.0	2.39	6.39	1.26	8.18
8	69	13.0	2.62	7.84	1.22	7.96
9	55	12.0	2.51	7.47	1.22	4.60
10	57	5.5	2.49	7.25	1.27	4.61
Mean	60.8	15.40	2.53	7.71	1.34	6.06
s.d.	11.90	9.04	0.06	0.82	0.19	2.87
Pony	n. MUP	%Poli.	Log ampl.	Dur.	Ar/Ampl	Fr
1	134	3.9	2.55	7.08	1.31	7.48
2	123	3.7	2.60	7.92	1.45	7.09
3	115	3.9	2.61	7.78	1.40	11.19
4	114	3.4	2.52	7.94	1.40	4.87
5	101	4.0	2.67	7.85	1.37	6.35
6	117	2.0	2.43	7.11	1.14	11.23
7	105	2.0	2.48	8.32	1.28	4.64
8	102	3.1	2.49	8.24	1.10	10.52
9	22	0.0	2.30	8.55	1.30	4.31
Mean	103.67*	4.81*	2.52	7.87*	1.31*	7.52*
s.d.	32.38	2.08	0.11	0.50	0.12	2.82

n.MUP, number of MUPs evaluated in the three muscles under study; % Poli., percentage of the number of polyphasic MUPs in confront of the totality of the MUPs, Log. ampl., logarithm of the amplitude; Dur., duration of the MUPs; Ar/Ampl., area/amplitude ratio; Fr, firing rate.
* $p < 0.05$ vs horses.

Platt, S.R., 2002. Advancing the role of electrodiagnostic techniques in equine neuromuscular disease. *Equine Veterinary Journal*, **34**(6), 538–539

Stalberg, E., Falch, B., Sonoo, M., Stalberg, S. and Astrom, M., 1995. Multi-MUP EMG analysis – a two year experience in daily clinical work. *Electroencephalography and Clinical Neurophysiology*, **97**, 145–154

Willison, R.G.J., 1964. Analysis of electrical activity in healthy and dystrophic muscle in man. *Neurology, Neurosurgery and Psychiatry*, **27**, 386–394

Wijnberg, J.D., Franssen, H., Van Der Kolk, J.H. and Breukink, H.J., 2002. Quantitative motor unit action potential analysis of skeletal muscles in the Warmblood horse. *Equine Veterinary Journal*, **34**, 556–561

Veterinary Research Communications, 28 (2004) 181–183

Pig Reactivity to Backtest and Growth During the First Three Months of Life

M. Faustini*, E. Munari, V. Russo, G. Pastorelli, G.L. Restelli, S. Scocca and D. Vigo
Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Facoltà di Medicina Veterinaria. Università degli Studi di Milano
**Correspondence: Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Facoltà di Medicina Veterinaria, Università degli Studi di Milano, Via Celoria,10, 20133 Milano, Italy*
E-mail: massimo.faustini@unimi.it

Keywords: swine, backtest, reactivity, growth

INTRODUCTION

Mammals react in a variable manner when stimulated by a stress or a novel situation through a behavioural coping reaction. Two main kinds of coping are known: a sympathetic reaction, with high general activity (active or proactive subjects), and a parasympathetic activity, with a general avoidance reaction, immobility and passivity (passive or reactive subjects) (Ruis *et al*., 2000).

In porcine species, individual responses can be related to genetics or direct learning, and the piglet's ability to react to a slight acute stress is easily determined by a backtest (BT) (Hessing *et al*., 1994). In the BT the subject is put on its back and constrained for one minute, counting the escape attempts. The relationship between BT and production vocation or immunology is controversial (Hessing *et al*., 1994; Jensen, 1995) and has not yet been fully clarified. In order to evaluate the relationships between reactivity and somatic growth, a backtest trial was performed taking into account some factors that could influence the subject's growth and relating these to body weight in the period from 3 to 90 days.

MATERIALS AND METHODS

The trial was performed in two Northern Italian piggeries in March-September on Landrace × Large White piglets from 38 litters. All piglets of each litter were considered. The total number of subjects was 342 at the start of the trial (3 days) and 121 at the end (3 months), with an equal proportion of males and females. The difference in number of piglets between the starting period and the end period is due to the fact that 200 animals were sold and 11 died during the trial. Piglets were treated with iron dextran (200 mg iron) and males were castrated at the age of 5–10 days. All piglets

were weaned at the age of 25–28 days. All piglets were individually weighed at day 3, 10, 17, 30 and 90 of life. Individual reactivity was assessed by a backtest (BT) (Hessing *et al*., 1994) on day 10 and 17 of life: briefly, the piglet is held on its back by an operator, who grabs the head (extended), and the hindlegs quite firmly for one minute. The escape attempts (wriggles separated by a pause) are counted. The total number of attempts for each test is employed to calculate a "reactivity class", as reported by van Erp-van der Kooij *et al*. (2000). Three classes are formed: high reacting piglets (HR), intermediate reactivity piglets (IR), and low reactive piglets (LR). In order to relate the reactivity class with the live weight, an ANOVA mixed model was performed: farm, sex, day and class were considered as fixed factors, whereas litter and piglet were considered as random effects, as reported below (random effects in bold):

$$\text{Weight}_{ijklmn} = \text{Farm}_i + \text{Sex}_j + \text{Day}_k + \text{ReactClass}_l + \mathbf{Litter}_m + \mathbf{Piglet}_n + \varepsilon_{ijklmn}$$

Multiple comparisons between groups were performed using a Tukey post-ANOVA test.

RESULTS AND DISCUSSION

On day 10 of life, the mean BT score was 2.09 ± 1.23, whilst on day 17, the mean BT score was 1.87 ± 1.19, with a total range of 0–6 attempts of escape/min. The percentage of piglets included in the LR class was 39.18%, while the IR class had the lowest percentage (24.56%). The HR class has an intermediate value, with a percentage of 36.26%. Descriptive statistics (expressed as mean value − standard deviation) and mixed model ANOVA results are reported in Table I. Results show that in the first three months of life there is an effect of the reactivity class on weight, in addition to that due to sex and time of weighing. HR piglets (with high reactivity) had a significantly lower ($p < 0.05$) weight with respect to the other two groups (5.8 ± 6.6 kg for the HR class versus 6.9 ± 8.3 kg for the LR class and 7.2 ± 9.2 kg for the IR class). It should be stressed that on day 3, the weight of the three groups did not differ.

These results confirm those obtained in a previous study (Faustini *et al*., 2003), where pre-weaning HR piglets showed lower body weight and growth. than LR or IR subjects.

These results are not in agreement with those reported by Van Erp-Van der Kooij *et al*. (2000) and Ruis *et al*. (2001), which did not show any effect of BT on growth perfomances, whilst Van Erp-Van der Kooij *et al*. (2000) observed a positive effect of the BT score on lean meat percentage.

These results contribute to a better characterization of a piglet's reactivity in response to a stressor. If the relationship between reactivity-growth versus composition were also confirmed for the latter phases of fattening, BT could be assumed as one of the parameters to permit the selection of animals with different growth-indices. In any case, a detailed analysis of the genetic effects of this aspect is necessary to confirm these preliminary results.

TABLE I
Mixed model (fixed factors only) ANOVA results for weight of piglets from 30 to 90 days

Factor level	Weight kg (mean ± sd)	p
Farm		
A	6.5 ± 7.4	n.s.
B	6.7 ± 8.7	
Sex		
M	5.8 ± 6.0	< 0.0001
F	7.4 ± 9.5	
Day		
3	2.1 ± 1.4	< 0.0001
10	3.5 ± 0.9	
17	4.9 ± 1.2	
30	7.4 ± 1.7	
90	30.6 ± 9.7	
Reactivity		
LR	6.9 ± 8.3[a]	< 0.05
IR	7.2 ± 9.2[a]	
HR	5.8 ± 6.6[b]	

[a,b] difference ($p < 0.05$) between groups.

REFERENCES

Faustini, M., Pastorelli, G., Munari, E., Restelli, G.L., Scocca, S., Colombani, C. and Maffeo, G., 2003. Caratterizzazione della reazione al backtest nel suinetto presvezzamento: correlazioni con l'accrescimento corporeo. *Atti della Società Italiana di Patologia ed Allevamento dei Suini, XXIX Annual Meeting*, Salsomaggiore Terme (PR), 27–28 marzo 2003, 235–243

Hessing, M.J.C., Hagelsø, A.M., Schouten, W.P.G., Wiepkema, P.R. and van Beek, J.A.M., 1994. Individual behavioural and physiological strategies in pigs. *Physiology & Behavior*, **55**, 39–46

Jensen, P., 1995. Individual variation in the behaviour of pigs – Noise or functional coping strategies? *Applied Animal Behaviour Science*, **44**, 245–255

Ruis, M.A.W., te Brake, J.H.A., van de Burgwal, J.A., de Jong, I.C., Blokhuis, H.J. and Koolhaas, J.M., 2000. Personalities in female domesticated pigs: behavioural and physiological indications. *Applied Animal Behaviour Science*, **66**, 31–47

Ruis, M.A.W., te Brake, J.H.A., Engel, B., Buist, W.G., Blokhuis, H.J. and Koolhaas, J.M., 2001. Adaptation to social isolation. Acute and long-term stress responses of growing gilts with different coping characteristics. *Physiology & Behavior*, **73**, 541–551

van Erp-van der Kooij, E., Kuijpers, A.H., Schrama, J.W., Ekkel, E.D. and Tielen, M.J.M., 2000. Individual behavioural characteristics in pigs and their impact on production. *Applied Animal Behaviour Science*, **66**, 171–185

Veterinary Research Communications, 28 (2004) 185–188

DNA Fingerprinting Using Microsatellites to Solve a Parentage Testing in the Boxer Breed

F. Gentilini[1]*, M.E. Turba[1] and G. Andreani[2]
[1] *Department of Veterinary Clinical Sciences, Internal Medicine Section, Faculty of Veterinary Medicine, University of Bologna, Ozzano Emilia, Italy;* [2] *Department of Biochemistry G. Moruzzi, Veterinary Section, Faculty of Veterinary Medicine, University of Bologna, Ozzano Emilia, Italy*
* *Correspondence: Dipartimento Clinico Veterinario, Sezione di Medicina Interna, Facoltà di Medicina Veterinaria, Alma Mater Studiorum Università di Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia, Italy*
E-mail: fgentilini@vet.unibo.it

Keywords: boxer, DNA, dog, fingerprinting, microsatellites, parentage test

INTRODUCTION

DNA microsatellites or Short Tandem Repeats (STRs) are short, tandemly repeated sequences of a bi-, tri- or tetranucleotide unit with a random distribution throughout the genome (Ostrander *et al*., 1995). They have been used extensively in applications as diverse as diagnosis of inherited diseases and forensic medicine for DNA fingerprinting and parentage testing. The success of this last application is due to the fact that STRs are highly polymorphic and, at the same time, they are sufficiently stable to be inherited unaltered from one generation to the next (Zajc *et al*., 1994; Ostrander *et al*., 1995; Muller *et al*., 1999). The aim of this work is to show an STR application to solve a doubtful paternity case for a boxer litter.

MATERIALS AND METHODS

Five puppies of the same litter, their mother and three putative sires were subjected to parentage testing. All subjects were boxer dogs. Among the three stud dogs, only one was seen to mate with the bitch two different times. Despite this, the breeder could not exclude undesired couplings involving the other two stud dogs. DNA was extracted from whole blood samples in K_3EDTA using a commercial available kit (Perfect Blood gDNA Mini Isolation Kit Eppendorf); genomic DNA was PCR amplified on Mini Cycler MJ Research thermalcycler. Ten microsatellites were selected from the literature; their flanking sequences or their specific amplification primer pairs were available alternatively. To our knowledge, allele frequencies and polymorphism specific for boxer breed microsatellites have never been investigated; therefore microsatellites were chosen based on high Heterozygosity (He) and

Polymorphism Information Content (PIC) values, previously calculated for other dog breeds, and product length suitable for the successive resolution on polyacrylamide gel (80–500 bp). The STRs selected were: AHT125, AHT140, AHT128, AHTf12, AHT121, Ren 02K21, Ren 05C07, FH 2001, FH2006, FH 2152 (Holmes *et al*., 1994; Holmes *et al*., 1995; Francisco *et al*., 1996; Holmes *et al*., 1998; Jouquand *et al*., 2000). Primer pairs from the literature were employed in all cases. Moreover, a primer pair for the microsatellite Ren 02K21 was designed by the authors on the basis of the known flanking sequences. Optimal conditions were established for each microsatellite by adjusting MgClθ, primer and template concentrations. The thermalcycling conditions were the same for all STRs tested. PCR products were run on 6% vertical polyacrylamide gel (Novex® TBE gel 6% Invitrogen; XCell Sure Lock™ MiniCell, Invitrogen) and revealed on a U.V. transilluminator after EtBr staining. Gel images were acquired using a digital camera. For each microsatellite, PCR products for all tested dogs were evaluated on the same gel while the size of the amplicons was estimated using a 50 bp ladder as reference standards (Step Ladder 50 bp, Sigma).

RESULTS

With the exception of microsatellite AHT f12, all STRs tested were correctly amplified with the conditions applied. Nevertheless, in the case of microsatellite Ren 02K21 only the primer pair designed by the authors, not those from literature, gave a specific PCR product. Six microsatellites, separated on polyacrylamide gel, did not show polymorphisms useful for the assignment of parentage. Three STRs (FH 2001, AHT121, Ren 02K21) revealed a full allele compatibility of all puppies with putative father no. 1, the only one who certainly mated the bitch, and excluded a possible superfecundation for puppies no. 1, 2 and 3. Furthermore, the exclusion of sire no. 2 from the paternity of puppy no. 4 was shown, while none of the possible fathers could be excluded from the paternity of puppy no. 5 because its allelic patterns were always compatible with those of all stud dogs (Fig. 1). DNA polymerase slippage made the allelic pattern interpretation more difficult in two cases. The thermalcycling conditions applied caused formation of a very large number of aspecific PCR products during amplification of microsatellite Ren 05C07.

DISCUSSION

Parentage testing interpretation relies on the fact that STRs are inherited in a true Mendelian fashion and express a codominant nature of allelic variants. Thus, a stud dog can be excluded as father of a litter when the puppies present one allele which could not have been inherited either from the bitch or the same stud dog. In the parentage testing presented, only putative sire no. 1 had full allele compatibility with all puppies. The visualisation of PCR products using polyacrylamide gel can reveal,

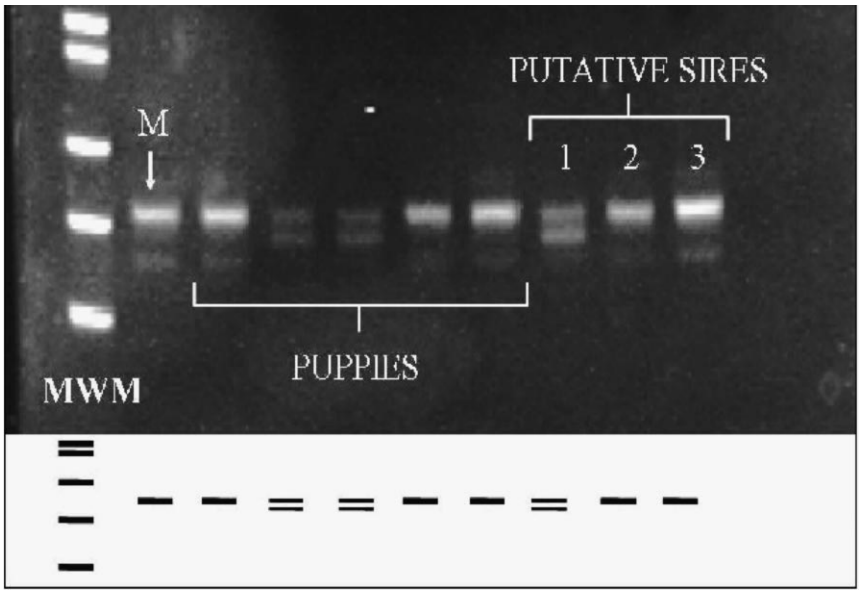


Figure 1. Interpretation of parentage testing using FH 2001 microsatellite. Mother (M) – Putative sire no. 1: fully compatible with all puppies. Mother (M) – Putative sire no. 2: exclusive compatibility with puppies no. 1, 4, 5. Mother (M) – Putative sire no. 3: exclusive compatibility with puppies no. 1, 4, 5

with some limitations, allele polymorphisms able to solve doubtful paternity cases. Nevertheless, in canine forensic medicine the case to solve often concerns possible mixed paternity, which means that puppies of the same litter may have been fathered by different sires (i.e. superfecundation). On this basis, the technology applied in this study allowed us to partially assign the paternity, leaving it unsolved for two of the five puppies. This may be explained by two considerations: the absence of literature data about microsatellite polymorphism in boxer dogs may have caused the poor choice of markers for solving parentage in this breed; moreover, the visualisation technology applied only allows us to distinguish alleles that differ greatly in size, at the same time reducing the potential information content of each microsatellite.

ACKNOWLEDGEMENTS

With technical support of Department of Veterinary Morphophysiology and Animal Production, University of Bologna, Ozzano Emilia, Italy.

REFERENCES

Binns, M.M., Holmes, N.G., Marti, E. and Bowen, N., 1995. Dog parentage testing using canine microsatellites. *Journal of Small Animal Practice*, **36**, 493–497

Francisco, L.V., Langston, A.A., Mellersh, C.S., Neal, C.L. and Ostrander, E.A., 1996. A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. *Mammalian Genome*, **7**(5), 359–362

Holmes, N.G., Strange, N.J., Binns, M.M., Mellersh, C.S. and Sampson, J., 1994. Three polymorphic canine microsatellites, *Animal Genetics*, **25**(3), 200

Holmes, N.G., Dickens, H.F., Parker, H.L., Binns, M.M. and Mellersh, C.S., 1995. Eighteen canine microsatellites, *Animal Genetics*, **26**(2), 132–133

Holmes, N.G., Dickens, H.F., Neff, M.W., Mee, J.M., Sampson, I. and Binns, M.M., 1998. Nine canine microsatellites, *Animal Genetics,* **29**(6), 477

Ichicawa, Y., Takagi, K., Tsumagari, S., Ishihama, K., Morita, M., Kanemaki, M., Takeishi, M. and Takahashi, H., 2001. Canine parentage testing based on microsatellite polymorphisms, *Journal of Veterinary Medical Science*, **63**(11), 1209–1213

Jouquand, S., Priat, C., Hitte, C., Lachaume, P., Andre, C. and Galibert, F., 2000. Identification and characterization of a set of 100 tri- and dinucleotide microsatellites in the canine genome, *Animal Genetics*, **31**(4), 266–272

Muller, S., Flekna, G., Muller, M. and Brem, G., 1999. Use of canine microsatellite polymorphisms in forensic examinations, *Journal of Heredity*, **90**(1), 55–56

Ostrander, E.A., Mapa, F.A., Yee, M. and Rine, J., 1995. One hundred and one new simple sequence repeat-based markers for the canine genome, *Mammalian Genome*, **6**(3), 192–195

Zajc, I., Mellersh, C., Kelly, E.P. and Sampson, J., 1994. A new method of paternity testing for dogs, based on microsatellite sequences, *The Veterinary Record*, **135**, 545–547

Veterinary Research Communications, 28 (2004) 189–192

Neurotrophins and the Thymus: Morphological Analysis of Mice Carrying a Non-functional Mutation on the Trka and Trkb Genes

A. Germanà*, R. Laurà, M.B. Levanti, G. Montalbano, G.P. Germanà, F. Abbate and E. Ciriaco
Dipartimento di Morfologia, Biochimica, Fisiologia e Produzioni Animali, Sezione di Morfologia, Università di Messina, Italy
**Correspondence: Dipart. di Morfologia, Biochimica, Fisiologia e Produzioni Animali – Sezione di Morfologia – Facoltà di Medicina Veterinaria – Università degli Studi di Messina – Polo Universitario dell'Annnunziata – Via SS. Annunziata – 98168 Messina – Italy*
E-mail: agermana@unime.it

Keywords: neurotrophins, knock-out mice, thymus, trk

INTRODUCTION

The family of trophic factors consisting of neurotrophins and their specific Trk receptors has been studied by many authors for their role at the level of the central and peripheral nervous system (see review by Huang and Reichardt, 2001). It has recently been demonstrated that these proteins also play an important role in the development and maintenance of the immune system. Data are present in the literature regarding the expression of neurotrophins and their receptors in the thymus of mammals, both at the level of mRNA and protein (Maroder *et al*., 1996), but data about the expression and localization of these factors in lymphoid organs still remain incomplete. Transgenic animals provide the most powerful way to demonstrate the effect of a protein *in vivo*. Mice with mutations targeted in genes codifying for the neurotrophins and Trk receptors provided important information about the role of these proteins in the development of the nervous system (Fariñas, 1999). Previous studies carried out using this experimental model demonstrated that the neurotrophins and Trk receptors play an important role in the development and maintenance of the thymic homeostasis (Aloe *et al*., 1999; Garcia-Suarez *et al*., 2000). Therefore, the aim of this study is to present the most recent data obtained in our laboratory and to clarify the possible functional meaning of the neurotrophins as modulators of the thymic microenvironment.

MATERIAL AND METHODS

Thymus of C57B1/6 mice, removed under deep chloral hydrate anesthesia (350 mg/Kh, i.p.) was processed for light microscopy, single and double immunohistochemistry, western-blot, RT-PCR and northern-blot analysis. The antibodies used for

immunohistochemical procedures were: anti-TrkA and anti-TrkB (Santa Cruz Biotechnology, CA, USA), anti-cytokeratins (DPC, Los Angeles, USA), anti-S100 (Dako, Copenhagen, Denmark), and anti-F4/80 (Serotec, Oxford, UK). For transmission electron microscopy analysis knock-out for *trkA* and *trkB* mice of the same stock from the colony of the Department of Molecular Biology, Bristol-Myers-Squibb Inc., Princeton, NJ (kindly provided by Dr. Silos-Santiago) was perfused transcardially with 2.5% glutaraldehyde. The thymuses removed were fixed in the same perfusion liquid, post-fixed in 1% osmium tetroxide, dehydrated and embedded in Durcupan® ACM (Fluka). Ultrathin sections were examined under a Jeol Jem 100 SX electron microscope.

RESULTS

Analysis of TrkA

The results obtained by Western-blot showed the presence of a proteinic band in the mouse thymus with a molecular weight of 140 kDa, corresponding to the full length TrkA receptor. The single and double immunohistochemistry showed that this protein was present in cells with cytoplasmic prolongations, localized at subcapsular and medullary levels. These cells were positive to cytokeratins, which are specific markers for epithelial cells, thus confirming their epithelial origin. The ultrastructural analysis of mouse thymus knock-out for TrkA showed clear morphological modifications at the medullary level, consisting of cysts of different sizes, lined by a monostratified epithelium which was often covered by cilia and containing scarce muciparous cells. The presence of these cysts reveals the persistence of a structure with embryonic origin.

Analysis of TrkB

The results obtained by Northern-blot demonstrated expression of the mRNA for TrkB in thymus of mice at different ages (0 and 15 days pn). An age-dependent decrease of the mRNA for thymus TrkB was observed, while this protein remained constant at the brain level used as a positive control. The analysis of the Western-blot carried out on samples of whole thymus, isolated lymphocytes, isolated stromal cells and brain of 15 day-old animals demonstrated, in all samples examined, the presence of a proteinic band of 145 kDa corresponding to the full length TrkB receptor. The immunohistochemical analysis revealed a strong immunoreactivity in stromal cells localized on the cortico-medullary border. The double immunohistochemistry for TrkB and F4/80 identified the non lymphoid cells positive to TrkB as macrophages. The ultrastructural study carried out on the thymus of TrkB knock-out mice demonstrated clear modifications in lymphocytes and stromal cells. The modifications of

lymphocytes consisted of fragmentation or absence of the nucleus and accumulation of cytoplasmic electrondense bodies (apoptotic bodies), as well as evident signs of apoptosis.

DISCUSSION

Neurotrophins are well known for their action on nervous tissues (Fariñas, 1999) but recent data have suggested that these proteins also act in the regulation of the immune system (Otten and Gadient, 1995; Aloe *et al*., 1999; Ruberti *et al*., 2000). Mice deficient in neurotrophins or those having non-functional neurotrophin receptors have added substantial information to the knowledge of the role of neurotrophins in nervous system development (Fariñas, 1999). However, the non-neuronal phenotype of these mouse strains has only been poorly analyzed, and remains basically unknown. Regarding TrkA deficient mice, we have demonstrated in this study that the lack of this protein provokes an altered organogenesis of the murine thymus. The thymus of TrkA knock-out mice showed the presence of cysts of different sizes lined by a single line of cells, cilia and mucous cells, probably due to the absence of *trka* functional genes. The mechanism through which the TrkA receptor could act on the development of thymic epithelial cells remains unclear. Regarding TrkB, the present study demonstrates that the thymus of mice lacking functional TrkB develops thymic alterations, at least in the cell population that normally expresses this receptor. The homozygous mice carrying a mutation in the *trkb* gene resulting in a non functional TrkB protein, showed structural and ultrastructural changes mainly consisting of massive cell death of T-lymphocytes for apoptosis. These data suggest that TrkB is involved in the prevention of the death of cortical lymphocytes. Our results are in agreement with previous data reported by Maroder *et al*. (1996), who observed that BDNF prevented cell death of immature thymocytes. Taken together, these results lend support to the idea that in the mammalian thymus both the NGF/TrkA and the BDNF/TrkB systems play important roles in the development and maintenance of epithelial cells and thymocytes, respectively, and that both ligand-receptor complexes are involved in the intercellular communication between these two main cell types of the thymus. This study on transgenic mice greatly contributes to the understanding of the possible role of neurotrophins in the immune system and the importance of these growth factors in health and disease.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. Jose Antonio Vega for reviewing the manuscript, to Dott. Olivia Garcia-Suarez for technical assistance and to Dott. Immaculade Silos-Santiago for providing Knock-out mice.

REFERENCES

Aloe, L., Simone, M.D. and Properzi, F., 1999. Nerve growth factor: a neurotrophin with activity on cells of the immune system. *Microscopy research and technique*, **45**(4–5), 285–291

Fariñas, I., 1999. Neurotrophin actions during the development of the peripheral nervous system. *Microscopy research and technique*, **45**(4–5), 233–242

Gadient, R.A. and Otten, U., 1995. Interleukin-6 and interleukin-6 receptor mRNA expression in rat central nervous system. *Annals of the New York Academy of Sciences*, Jul 21; **762**, 403–406

Garcia-Suarez, O., Germanà, A., Hannestad, J., Ciriaco, E., Laura, R., Naves, J., Esteban, I., Silos-Santiago, I. and Vega, J.A., 2000. TrkA is necessary for the normal development of the murine thymus. *Journal of Neuroimmunology*, **108**(1–2), 11–21

Huang, E.J. and Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. *Annual Review of Neuroscience*, **24**, 677–736

Maroder, M., Bellavia, D., Meco, D., Napolitano, M., Stigliano, A., Alesse, E. Vacca, A., Giannini, G., Frati, L., Gulino, A. and Screpanti, I., 1996. Expression of TrkB neurotrophin receptor during T cell development. Role of brain derived neurotrophic factor in immature thymocyte survival. *Journal of Immunology*, **157**(7), 2864–2872

Ruberti, F., Capsoni, S., Comparini, A., Di Daniel, E., Franzot, J., Gonfloni, S., Rossi, G., Berardi, N. and Cattaneo, A., 2000. Phenotypic knockout of nerve growth factor in adult transgenic mice reveals severe deficits in basal forebrain cholinergic neurons, cell death in the spleen, and skeletal muscle dystrophy. *Journal of Neuroscience*, **20**(7), 2589–2601

Veterinary Research Communications, 28 (2004) 193–196

Immunodiscrimination Between Native and Recombinant Somatotropin: a Possible Pathway?

A. Guidi[1]*, G. Iannone[2], L. Castigliego[1] and D. Gianfaldoni[1]
[1] *Department of Animal Pathology Prophylaxis and Food Hygiene, Faculty of Veterinary Medicine, University of Pisa, 56100 Pisa, Italy;* [2] *Institute of Biomedical Technologies, CNR Pisa Italy*
* *Correspondence: Department of Animal Pathology Prophylaxis and Food Hygiene*
E-mail: aguidi@vet.unipi.it

Keywords: BST, pharmacological treatment, polyclonal antibodies

Abbreviations: ST, somatotropin; rBST, recombinant bovine somatotropin; IGF-1, insulin like growth factor 1; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin

INTRODUCTION

Somatotropin (ST) is a peptidic hormone produced by the pituitary gland in mammals which performs regulative functions in growth and lactation in ruminants.

The recombinant form of bovine ST (rBST) is used to increase milk yield in cows. This kind of treatment, which is authorised in many countries, has been definitively banned by the E.U. because of its potential hazard to animal health (Kronfeld, 2001; Macrì, 1999). There are currently no analytical methods able to reveal rBST administration. However, there are circulating molecules which can be considered as analytical targets and indirect indicators for the abovementioned treatment of bovines, since their concentration is proportional to the stimulative action of ST. The most famous of these is Insulin-like Growth Factor 1 (IGF-1), a hormone mainly secreted by the liver (Prosser *et al*., 1989) that mediates the biological action of ST on several tissues. Almost all seric IGF-1 circulates bound to specific IGF-binding proteins (IGFBPs); for this reason the current analytical methods used to measure it are still imprecise (Breier *et al*., 1991; Twigg *et al*., 2000).

On the other hand, the direct analytical approach is very difficult because the primary sequence of recombinant BST differs from the native form for only one aminoacid at the N-terminal position.

The aim of our work is to evaluate the possibility of producing antibodies capable of direct discrimination, between BST and rBST. We have used the murine immune system as our model.

We produced polyclonal antibodies against a synthetic peptide that mimics the N-terminal region of rBST. Immunoenzymatic tests of the sera derived from immunised animals allowed us to evaluate their eventual different reactivity against the two forms of the protein, suggesting the possibility of producing monoclonal antibodies

with a higher affinity for the recombinant form. Immunoglobulins with these characteristics are determinants for the development of analytical methods able to reveal the rBST pharmacological treatment.

MATERIALS AND METHODS

With the aim of obtaining antibodies against the N-terminal region of rBST, balb-c mice were immunised with a complex formed from a peptide conjugated to BSA or KLH, used as a carrier protein (BSA-Peptide and KLH-Peptide). The peptide sequence corresponds to the first ten rBST N-terminal amino acids, followed by a tail of lysines and serines to augment water solubility: H_3N--M-A-F-P-A-M-S-L-S-G-S-S-K-K-C--COOH.

Five mice were treated with BSA-peptide and another five with KLH-peptide. Two immunisation cycles were performed using 50 μg of the peptide conjugated in Freund's adjuvant (complete for the first cycle, incomplete for the following 3 boosts) administered every two weeks subcutaneously. Then, after confirmation of the antibody titre, mice were sacrificed for bleeding.

Antigens and analytical methods: BST, rBST, BSA-peptide, KLH-Peptide, and BSA, KLH and myoglobin, used a as control antigen, were utilised at different concentrations to evaluate the specific antibody titre of the sera. ELISA and Western blots were utilised as immunoenzymatic tests.

Direct ELISA were performed by incubating sera at various dilutions with the antigen bound to the microtitration plate and polyclonal anti-mouse total IgG conjugated with alkaline phosphatase was used for the binding revelation. The electroblotting first required a SDS-Page antigen separation and their subsequent transfer onto nitrocellulose membranes. Membranes were there incubated with immune sera and the final revelation was performed as previously described for ELISA.

RESULTS

In both groups of mice, the immunoenzymatic tests revealed a much higher antisera avidity toward carrier-peptide complexes than toward the two species of ST, even when the peptide was conjugated to a different carrier than the one employed for the immunization (Fig. 1). This evidence can be explained by considering that only a fraction of the antibodies developed against the peptide is also able to recognize the BST head, namely those directed against the first amino acids in the N-terminal position of the protein: especially those presenting an intrinsic steric adaptability. Specifically comparing the reactivity of the whole sera on the two types of BST, we observed a positive reaction for both of them in eight out of the ten analyzed mice. The fact that some mice did not produce an appreciable immune response against the BST N-terminal portion could be explained by considering the remarkable similarity

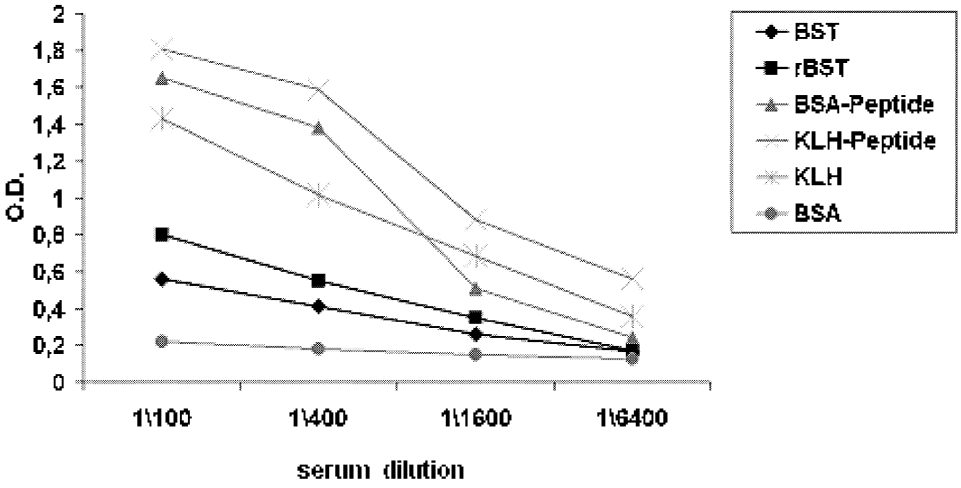


Figure 1. Mean values of ELISA performed with the sera of the three best mice immunised with KLH which showed a higher avidity to rBST as compared to BST

between the ST of bovines and that of rodents. Within the group of responsive mice, the reaction of the antibodies was comparable for both molecular forms in four animals (three of which had been immunized with BSA-peptide), whereas the other four (three of which had been immunized with KLH-peptide) showed visibly higher reactivity against the recombinant form.

DISCUSSION

These results suggest a possible proliferation of lymphocytic clones producing immunoglobulins with a higher affinity for the recombinant form of ST. Consequently, we consider the efforts aimed at producing monoclonal antibodies with these characteristics, using the lymphopoietic tissues derived from animals with a higher discriminative response, to be a valid approach to this problem.

REFERENCES

Bauman, D.E., Everett, R.W., Weiland, W.H. and Collier, R.J., 1999. *Journal of Dairy Science*, Dec., **82**, 2564–2573.

Breier, B.H., Gallaher, B.W. and Gluckman, P.D., 1991. Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls .*Journal of Endocrinology*, **128**, 347–57.

Kronfeld, D.S., 2001. Concerned about use of BST in dairy cattle. *Journal of American Veterinary Medicine Association*, Sep 1, **219**(5), 591–592.

Macrì, A., 1999. La posizione dei comitati scientifici della U.E. sulla somatotropina bovina ricombinante. *Il Nuovo Progresso Veterinario*, **54**, 529–530.

Prosser, C.G., Fleet, I.R. and Corps, A.N., 1989. Increased secretion of insulin-like growth factor I into milk of cows treated with recombinantly derived bovine growth hormone. *Journal of Dairy Research*, **56**, 17–26.

Twigg, S.M., Hardman, K.V. and Baxter, R.C., 2000. A purified bovine serum albumin preparation contains an insulin-like growth factor (IGF) binding protein-3 fragment that forms ternary complexes selectively with IGF-II and the acid-labile subunit. *Growth Hormone and IGF Research*, Aug., **10**, 215–223.

Veterinary Research Communications, 28 (2004) 197–200

Occurrence of Two Different Mechanisms of Apoptosis in Cerebellar Granule Cells in Relation to the Specificity of Poly-ADP-ribose Polymerase-1 (PARP) Activation

L. Lossi
Dipartimento di Morfofisiologia Veterinaria-Facoltà Medicina Veterinaria. Via. L.da Vinci 44 I-10095 Grugliasco (To), Italy
E-mail: laura.lossi@unito.it

Keywords: cerebellum, granule cells, apoptosis, caspases, checkpoint proteins

INTRODUCTION

Programmed cell death (PCD) is an essential process in development that often occurs through activation of an apoptotic mechanism. Apoptosis can be detected in numerous areas of the central nervous system (CNS) under normal conditions but also in several neurodegenerative diseases. In the postnatal cerebellum (Lossi *et al*., 2002) apoptosis affects mainly pre-migratory granule cells (CGCs), their precursors within the external granular layer (EGL), and post-mitotic, post-migratory CGCs within the internal granular layer (IGL).

Apoptosis is characterized morphologically by a series of nuclear alterations including fragmentation and chromatin condensation eventually leading to the appearance of the so called apoptotic bodies, consisting of highly condensed cellular and nuclear fragments. Numerous proteins are involved in the execution of apoptosis, among which caspases play a pivotal role (Allsopp *et al*., 2000). Caspases are synthesized as inactive precursors that are activated following proteolysis. In CGCs, caspase 3 is activated at the endpoint of a cascade of events which affects other members of this protein family. The main substrate of active caspase 3 is poly-ADP-ribose-polymerase-1 (PARP-1 – Fig. 1A), a protein normally involved in genomic surveillance and DNA reparation (Smith, 2001). Following caspase 3 cleavage, a 85 kD fragment (p85) is generated that is known to be responsible for at least some of the morphological changes of apoptosis.

Other proteins involved in the regulation of apoptosis are related to cell cycle progression and checkpoint: these include the retinoblastoma protein (Rb) and several kinases that control the transition between the various phases of the cell cycle depending upon their degree of phosphorylation (Padmanaban *et al*., 2001).

In the current work we have studied the distribution of p85 and some cell cycle-

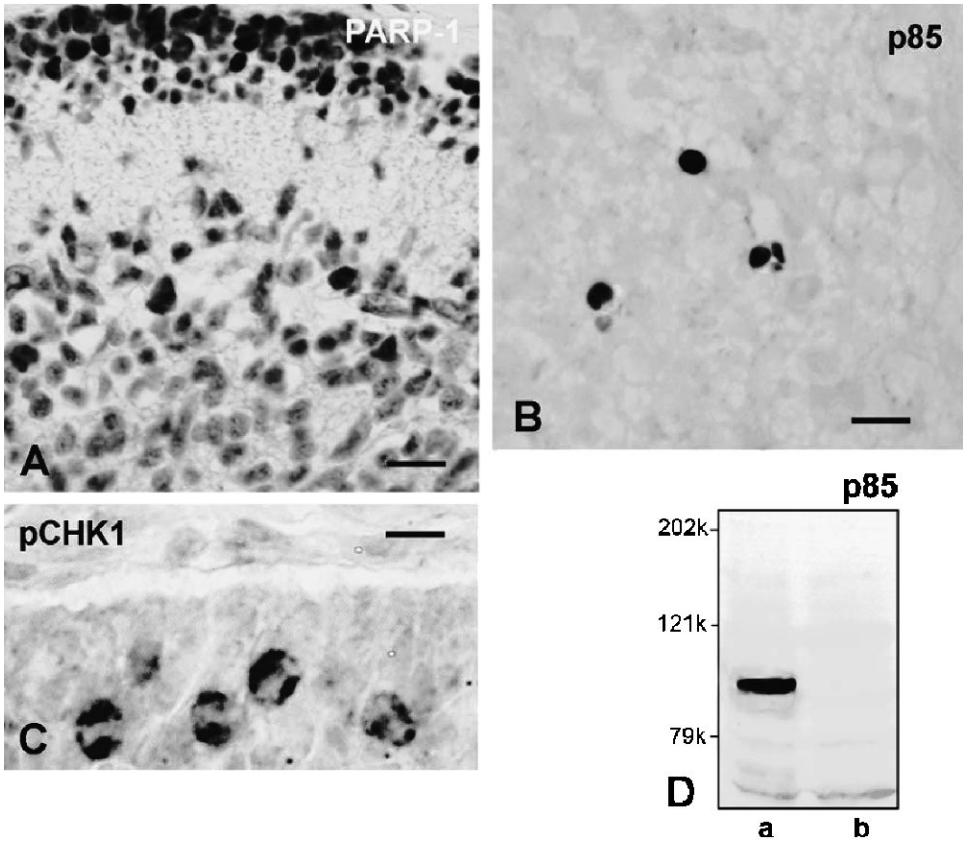


Figure 1. A: PARP-1 shows a virtually ubiquitous distribution within the cerebellar cortex; B: p85, the cleaved fragment of PARP-1, is only detected in isolated IGL cells; C: phospho-Chk1 is localized exclusively within the CGCs of the EGL; D: Western blotting of P5 cerebellar (a) and cerebral (b) extracts showing expression of p85 in the cerebellum. Bars: A-B: 10 μm; C: 5 μm

related proteins in the post-natal cerebellum to shed some light on the interaction between these molecules in the regulation of the apoptotic process affecting CGCs.

MATERIALS AND METHODS

Cerebella were obtained from New Zealand rabbits at postnatal day 5 (P5) after vascular perfusion with 4% paraformaldehyde. Parasagittal sections were labeled using the TUNEL method for *in situ* detection of DNA fragmentation and the ABC

procedure for immunocytochemical detection of cleaved caspase 3, p85, phospho-Rb (ser780), phospho-Rb (ser795), phospho-Chk1, and phospho-cdc2 (Tyr15).

The presence of p85 was also assessed in Western blotting from cerebellar extracts.

RESULTS

Within the EGL there is no positive stain for cleaved caspase 3 or p85. In this layer of the forming cerebellar cortex we observed a positive immunoreaction for phospho-Rb, which is responsible for blocking the G_1/S transition of the cell cycle, at the level of premigratory CGCs. Within the EGL there is also a positive stain for phospho-Chk1, which blocks the cell cycle at G_2/M. Cells with the cytoplasmic stain for cdc2 correspond to proliferating neurons. Within the IGL there is no expression of these cell cycle-related proteins, whereas a positive signal for cleaved caspase 3 and p85 was detected, with a cell distribution overlapping that of TUNEL positive nuclei (Fig. 1B-D).

DISCUSSION

This work demonstrates that in the postnatal cerebellum two different apoptotic pathways occur, affecting the CGCs at different stages of their maturation. In the IGL the apoptotic pathway is "classically" executed by caspases, with cleavage of caspase 3 and PARP-1 as the major endpoint events. In the EGL apoptosis appears to be caspase-independent and involves the activation/inactivation of certain checkpoint proteins and, most likely, some mitochondrial factors such as the apoptosis-inducing factor (AIF)(Susin *et al*., 1999). AIF is a recently discovered molecule that is normally localized inside mitochondria, but, following an apoptotic stimulus, it translocates to the nucleus, where it triggers some of the morphological alterations typical of programmed cell death.

ACKNOWLEDGEMENTS

This work was supported by local funds (Fondi ex60% 2002) from the University of Torino. I wish to thank prof. A. Merighi for useful scientific discussion.

REFERENCES

Allsopp, T.E., McLuckie, J., Kerr, L.E., Macleod, M., Sharkey, J. and Kelly, J.S., 2000. Caspase 6 activity initiate caspase 3 activation in cerebellar granular cell apoptosis. *Cell Death and Differentiation*, **9**, 984–993

Lossi, L., Mioletti, M. and Merighi, A., 2002. Synapse-independent and synapse-dependent apoptosis of

cerebellar granule cells in postnatal rabbits occur at two subsequent but partly overlapping developmental stages. *Neuroscience*, **112**, 509–523

Padmanabhan, J., Park, D.S., Greene, L.A. and Shelanski, M.L., 1999. Role of cell cycle regulatory proteins in cerebellar granule neuron apoptosis. *Journal of Neuroscience*, **19**, 8747–8756

Smith, S., 2001. The world according to PARP. *Trends Biochemical Science*, **26**, 174–179

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G., 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*, **397**, 441–446

Veterinary Research Communications, 28 (2004) 201–204

2B Myosin Heavy Chain Isoform Expression in Bovine Skeletal Muscle

F. Mascarello*[1], L. Maccatrozzo[1], M. Patruno[1], L. Toniolo[2] and C. Reggiani[2]
[1] *Dipartimento di Scienze Sperimentali veterinarie;* [2] *Dipartimento di Anatomia e Fisiologia Umana, Università di Padova, Italy*
* *Correspondence: Facoltà di Medicina Veterinaria, Agripolis, Dipartimento di Scienze Sperimentali veterinarie, Viale dell'Università 16, 35020,-Legnaro (PD), Italy*
E-mail: masca@unipd.it

Keywords: bovine, skeletal muscle, 2B MHC, RT-PCR, electrophoresis, cDNA

INTRODUCTION

Sarcomeric myosin is characterised by a high structural heterogeneity which is essential to achieve different functional performances. This variability is based on the presence of different myosin heavy chain isoforms (MHC) which are considered to be the marker of fibre types.

Different genes that codify for type 1, 2A, 2X and 2B myosin heavy chain isoforms (paralogous isoforms) present in skeletal muscle have been identified. Furthermore, there are also less frequent isoforms which are expressed in some special muscles or transitionally expressed during development. When corresponding isoforms from different species are compared (orthologous isoforms) it is possible to detect a low nucleotidic variability that determines the different contractile performances of muscle fibres.

In muscle, "pure" fibres express a single myosin heavy chain isoform whereas "hybrid" fibres express, in the same fibre, more than one MHC isoform: for example type 1/2A, (conventionally called 2C), 2A/X and 2X/B.

The isoform type 2B (2B fibre type) has been detected only in small mammals (mouse, rat, rabbit and marsupials) and is the most represented isoform in some muscles.

This isoform is not expressed in humans although the gene is present. Moreover, the fibre type originally classified histochemically as 2B in humans shows a higher similarity to the 2X of rat and mouse than to the 2B of the same species. However, Bottinelli and Reggiani (2000) have shown that in man, where only three MHC isoforms (type 1, 2A and 2X) are present, the functional plasticity of muscle is not lost when compared to small mammals in which the 2B isoform is also expressed.

As far as other species (dog, cat, horse, monkey, goat) are concerned, recent studies have identified only three type of fibres or three isoforms as for humans. Two of these isoforms (type 1 and type 2A) are very similar to the rat and mouse isoforms while the third one seems to be more similar to 2X. The general opinion among researchers

is that the 2B type would be specific only for small mammals and marsupials. The latter hypothesis has not been confirmed for the pig where the 2B isoform is present and is expressed in several muscles and, as shown by our research, is in association with the isoform 2X (hybrid fibres 2X/B).

In bovine, histochemical, immunohistochemical and electrophoretic data have identified three isoforms, indicated as type 1, 2A and 2B (Duris *et al*., 2000); these data have been only partially confirmed by gene expression studies where the 1, 2A and 2X isoforms have been identified (Tanabe *et al*., 1998).

Since the isoform 2B might also be expressed in large animals, the aim of this study was to assess the presence of the gene codifying the 2B isoform, its expression by RT-PCR and the presence of the corresponding protein by gel-electrophoresis.

MATERIALS AND METHODS

Samples were dissected from bovine muscles with different composition of fibre types, such as masseter, longissimus, extensor carpi radialis, diaphragma, pectoral, extraocular muscles (rectus lateralis) and retractor bulbi.

Gene isolation

Genomic DNA was extracted from bovine blood and two regions of 2B isoform gene were amplified using degenerate primers, designed on the basis of known sequences (man and pig). The two isolated fragments were cloned and sequenced.

RT-PCR

Total RNA was extracted from dissected muscles and reverse transcribed using a reverse transcriptase enzyme. Based on our genomic results, bovine 2B MHC specific primers were designed, while specific oligonucleotides to amplify the type 1, 2A and 2X MHC isoforms were designed on bovine sequences data present in literature.

Electrophoresis

Fragments of the above described sampled muscles were solubilised in Laemmli solution. Proteins were separated on 9% polyacrylamide slab gels following a procedure developed by Blough *et al*. (1996) by modifying electrophoretic running parameters. Electrophoresis was run at constant 50 Volt for 2 h followed by 40 h at 200 V at 4°C. Electrophoretic bands in the migration zone of the myosin heavy chain

(200 kD) were identified by comparison with samples previously analysed by RT-PCR.

RESULTS

Two fragments of 2B isoform were sequenced from genomic bovine DNA. The first one (123 nt, GenBank AY135646) corresponding to the human exon 40, shows a strong similarity (96%) to the 2X and 2B and therefore seems inadequate for a precise identification of 2B gene. The second fragment (515 nt GenBank AY227972) was amplified with a forward primer designed on the loop2 exon 16 (a region that presents the higher variability between paralogus isoforms) and with a reverse primer on exon 21. The sequence obtained from the second fragment is more similar to 2B than to 2X published sequences. The nucleotidic identity score is 93% with 2B isoform of man and pig, while the nucleotidic identity score with 2X isoform is 90% (man) and 91% (pig and bovine). The phylogenetic tree confirms the position of our sequence in the orthologous 2B group.

RT-PCR analysis shows the expression of the type 1 isoform in masseter muscle, of type 1 and 2A in diaphragma, and of type 1, 2A and 2X in longissimus, pectoral, extensor carpi radialis and extraocular muscles. In all muscles examined only the rectrator bulbi and the rectus lateralis express the type 2B isoform together with type 1, 2A and 2X.

Protein electrophoretic separation shows the same pattern of expression observed by RT-PCR. Comparing muscles with different composition of fibre types allowed precise identification of the electrophoretic bands of type 1, 2A and 2X. The band that corresponds to 2B isoform is difficult to identify since its electrophoretic migration is similar to that of the extraocular band, a MHC typical of rotator muscles (such as the rectus dorsali) but not or scarcely present in retractor bulbi (Sartore *et al*., 1987).

DISCUSSION

Our results show that the 2B MHC gene is present in the bovine genome. However, skeletal muscles express only type 1, 2A and 2X MHC. The pig is therefore the only species where 2B isoform is expressed in skeletal muscles (Chikuni *et al*., 2001) although often in association with the isoform 2X (hybrid fibres). In man, recent data indicate the expression of 2B mRNA in some particular muscle such as the masseter but the corresponding protein has not been detected.

In bovine, only a very peculiar muscle such as the retractor bulbi, shows expression of 2B and the corresponding protein shows an electrophoretic migration velocity very similar to the extraocular isoform. Therefore, it will be important to improve the SDS-gel electrophoresis technique in order to distinguish these two bands. It is difficult to explain the presence of this isoform in the retractor bulbi in view of its poor function and the disappearance of this muscle in man.

REFERENCES

Bottinelli, R. and Reggiani, C., 2000. Human skeletal muscle fibres: molecular and functional diversity. *Progress in Biophysics and Molecular Biology*, **73**, 195–262

Blough, E.R., Rennie, E.R., Zhang, F. and Reiser, P.J., 1996. Enhanced electrophoretic separation and resolution of myosin heavy chains in mammalian and avian skeletal muscles. *Analytical Biochemistry*, **233**, 31–35

Chikuni, K., Tanabe, S., Muroya, S. and Nakajima, I., 2001. Differences in molecular structure among porcine myosin heavy chain 2a, 2x and 2b isoforms. *Meat Science*, **57**, 311–317

Duris, M., P., Picard, P. and Geay, Y., 2000. Specificity of different anti-myosin heavy chain antibodies in bovine muscle. *Meat Science*, **55**, 67–78

Sartore, S., Mascarello, F., Rowlerson, A., Gorza, L., Ausoni, S., Vianello, M. and Schiaffino, S., 1987. Fibre types in extraocular muscles: a new myosin isoform in the fast fibres. *Journal of Muscle Research and Cell Motility*, **8**, 161–172

Tanabe, R., Muroya, S. and Chikuni, K., 1998. Sequencing of the 2a, 2x and slow isoforms of the bovine myosin heavy chain and the different expression among muscles. *Mammalian Genome*, **9**, 1056–1058

Veterinary Research Communications, 28 (2004) 205–208

Oocyte Maturation is Required for Correct Sperm Chromatin Rearrangement

M. Mattioli*, L. Gioia, M. Turriani, G. Capacchietti, P. Loi and G. Ptak
Dipartimento di Scienze Biomediche Comparate – Sezione di Fisiologia – Facoltà di Medicina Veterinaria – Università di Teramo, Italy
**Correspondence: Dipartimento di Scienze Biomediche Comparate – Sezione di Fisiologia – Facoltà di Medicina Veterinaria – Università di Teramo, Italy*
Piazza Aldo Moro 45, 64100 TERAMO
E-mail: mattioli@ifv.vet.unite.it

Keywords: acetylation, chromatin, methylation, remodelling, zygote

INTRODUCTION

Embryo development is dependent on the complex control of gene expression that is strictly regulated through specific rearrangements of maternal and paternal chromatin after fertilization. Most of the epigenetic changes of the genome structure are realized at an early stage after fertilization and include mechanisms of DNA methylation and histone acetylation. This process of genome remodelling seems to be possible thanks to the cytoplasmic machinery prepared by the oocyte during its maturation, since incomplete oocyte maturation, as often occurs following *in vitro* protocols, results in defects in gene expression and leads to abnormal embryo development (Young, 2000). On this basis the present research has been designed to describe and compare the epigenetic modifications (methylation/acetylation) that take place in pig zygotes obtained by fertilizing oocytes matured *in vivo* or *in vitro*.

MATERIAL AND METHODS

For this research zygotes produced *in vivo* or *in vitro* were utilized. For *in vivo* experiments prepubertal gilts were injected with 1250 IU of eCG, to induce follicular growth and maturation and, 60 h later, with 750 IU of hCG to induce ovulation within 40–44 h. 30 h later the hCG gilts were inseminated with semen obtained from boars of proven fertility and the zygotes were collected after 10–12 h by uterine flushing carried out under anaesthesia. For embryos produced *in vitro*, COCs isolated from healthy follicles, 4–5 mm in diameter, were matured in tissue culture medium (TCM) 199 with the support of both the gonadotropins (1 μg/ml USDA-pLH-B1 and 1 μg/ml USDA-pFSH-B1) for 40 h, as previously described (Mattioli, 1994). The *in vitro* fertilization (IVF) protocol was carried out by co-incubating denuded oocytes for 2 h with capacitated spermatozoa at a concentration of 2×10^6 spz/ml. Fresh

semen from boars of proven fertility was used for the IVF procedure. Oocytes were then transferred to modified TCM 199 (Mattioli, 1989) and incubated for a further 8–10 h to reach the pronuclei stage.

In order to evaluate and compare the pattern of DNA methylation and histone acetylation, the zygotes, produced either *in vivo* or *in vitro* at the same developmental stage, were utilized for the immunofluorescence protocol. To this end the zygotes were briefly incubated in Tyrode's solution and transferred to 0.1% pronase to remove the zona pellucida, washed with 0.05% Tween-20 in PBS, fixed for 1 h in 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature.

For the detection of global DNA methylation, zygotes were treated with 2M HCl at room temperature for 30 min to effect DNA denaturation and a specific primary antibody, mouse monoclonal anti-5-methyl-cytosine (dilution 1:500), was used.

For the detection of global histone acetylation fixed and permeabilized zygotes were immediately incubated with the primary antibody, an anti-hyperacetylated histone H4 (dilution 1:300). For both the methylation and acetylation protocols the primary antibody was detected by the specific secondary antibody coupled with Cy-3 (dilution 1:400). Negative control samples, in which the primary antibody was omitted, were also evaluated for each experiment.

At the end of the immunofluorescence protocol zygotes were analyzed using a Bio-Rad confocal laser scanning microscope equipped with a krypton/argon ion laser.

Digital optical sections were obtained by scanning a z series of 0.2 μm slices throughout the planes of focus containing maternal and paternal pronuclei and the images were later projected.

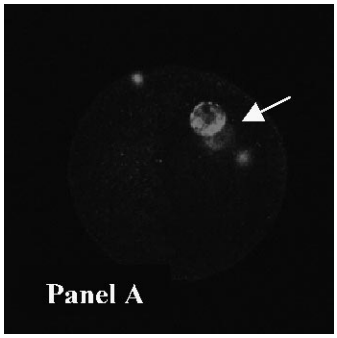
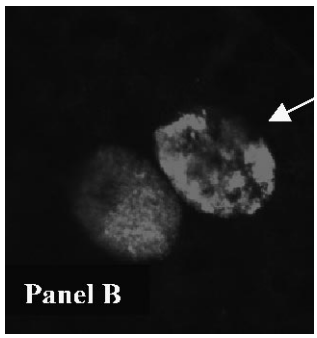
Identification of the male pronucleus

Some zygotes were fixed in acetic acid/ethanol (1:3) for 24 h, permeabilized and processed both for staining with lacmoid and observation under a microscope equipped with phase contrast, in order to identify the male pronucleus by the presence of the sperm tail nearby, and then for the immunofluorescence protocol and confocal microscopy analysis for evaluation of DNA methylation and histone acetylation.

RESULTS

The evaluation of the global methylation pattern of DNA in pig zygotes obtained *in vivo* showed that in most of the zygotes (79%, $n = 39$) maternal and paternal pronuclei showed a different pattern of methylation. The male pronucleus resulted frequently demethylated (Table I, panel A), in agreement with results obtained for other species (mouse, rat and bovine; Mayer, 2000; Dean *et al.*, 2001). The global methylation pattern of DNA evaluated in pig zygotes produced *in vitro* showed that only 40% ($n = 45$, $p < 0.05$) revealed demethylation of the paternal genome (Table I).

TABLE I
Pattern of DNA methylation and histone acetylation in pig zygotes produced from oocytes matured and fertilized *in vivo* or *in vitro*

DNA methylation		Histone acetylation	
	% of demethylated male PN		% of hyperacetylated male PN
Zygotes produced *in vivo*	79%, $n=39$	Zygotes produced *in vivo*	87%, $n=41$
Zygotes produced *in vitro*	40%*, $n=45$	Zygotes produced *in vitro*	67%*, $n=45$

Panel A: DNA methylation in parental pronuclei: male genome (arrow) more demethylated as compared to the female one.
Panel B: histone acetylation in parental pronuclei: male genome (arrow) hyperacetylated and its pattern of fluorescence characterized by the presence of clusters, in contrast with the more homogeneous distribution observed in the female pronucleus.
*Indicates data significantly different ($p < 0.05$).

The analysis of global histone acetylation in zygotes produced *in vivo* demonstrated that, in addition to a lower degree of DNA methylation, most of the male pronuclei (87%, $n = 41$) also showed a global condition of hyperacetylation of histone H4 as compared to the female pronuclei (Table I, panel B), with the male pronucleus characterized by clusters of chromatin asymmetrically distributed inside it, as compared to the female pronucleus where the fluorescence was homogeneously distributed.

The same evaluation carried out for zygotes produced *in vitro* showed that only 67% of male pronuclei were hyperacetylated ($n = 45$; $p < 0.05$), also in this case showing a reduced and slowed ability of oocytes to remodel the paternal genome. In addition, the morphology of the pronuclei and the pattern of global DNA acetylation was often more irregular as compared to that of oocytes matured *in vivo*.

DISCUSSION

The results presented in the present paper show that a process of chromatin remodelling takes place in pig oocytes during the early hours following fertilization. This is

mainly due to epigenetic modifications, such as DNA methylation and histone acetylation, and was found to be differentiated between maternal and paternal pronuclei. The analysis at a global level of DNA methylation, first carried out in zygotes produced *in vivo*, has in fact demonstrated that the male genome is characterized by a lower level of DNA methylation as compared to the female one. Moreover, the observation that the paternal pronucleus was also characterized by a higher level of histone acetylation in most of the zygotes analysed seems to indicate an early transcription of the male chromatin component. The same analysis of DNA methylation and histone acetylation subsequently carried out in pig zygotes obtained from oocytes matured and fertilized *in vitro*, demonstrated that the ability to correctly remodel the sperm chromatin is markedly reduced when compared to that of zygotes obtained *in vivo*. This demonstrated that the process of oocyte maturation is crucial for the oocyte to develop the mechanisms necessary for complete chromatin rearrangement. In fact, when the process of maturation doesn't occur under physiological conditions, such as in the case of maturation *in vitro*, this results in a reduced ability to correctly remodel the paternal genome that may compromise the success of embryo development.

ACKNOWLEDGEMENTS

This work was supported by es. Fin. M.I.U.R. 2001.

REFERENCES

Leighton, P.A., Ingram, R.S., Eggenschwiler, J., Efstratiadis, A. and Tilghman, S.M., 1995. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature*, **375**, 34–39

Mattioli, M., Bacci, M.L., Galeati, G. and Seren, E., 1989. Developmental competence of pig oocyte matured and fertilized *in vitro*. *Theriogenology*, **31**, 1201–1207

Mattioli, M., Galeati, G., Barboni, B. and Seren, E., 1994. Concentration of cyclic AMP during the maturation of pig oocyte *in vivo* and *in vitro*. *Journal Reproduction Fertility,* **100**, 403–409

Mattioli, M., Barboni, B., Lucidi, P. and Seren, E., 1996. Identification of capacitation in boar spermatozoa by chlortetracycline staining. *Theriogenology*, **45**, 373–381

Mayer, W., Niveleau, A., Walter, J., Fundele, R. and Haaf, T., 2000. Demethylation of the zygotic paternal genome. *Nature*, **403**, 501–502

Surani, M.A., 2001. Reprogramming of genome function through epigenetic inheritance. *Nature*, **414**, 122–128

Young, L.E. and Fairburn, H.R., 2000. Improving the safety of embryo technologies: possible role of genomic imprinting. *Theriogenology*, **53**, 627–648

Veterinary Research Communications, 28 (2004) 209–212

Inhaled Carbon Monoxide (CO) Prevents Lung Oedema Induced by Endotoxic Shock

S. Mazzola[1], M. Forni[2], M. Albertini[1]*, M.L. Bacci[2], B. Ciminaghi[1], M. Lavitrano[3], E. Seren[2] and M.G. Clement[1]

[1] *Department of Animal Pathology, Hygiene and Public Veterinary Health, Section of Biochemistry and Physiology, University of Milan, Italy;* [2] *Department of Veterinary Morphophysiology and Animal Production, University of Bologna, Italy;* [3] *Department of Experimental Environmental Medicine and Medical Biotechnology, University of Milano-Bicocca, Italy*

* *Correspondence: Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria – Sezione di Biochimica e Fisiologia Veterinaria – Facoltà di Medicina Veterinaria – Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy*
E-mail: mariangela.albertini@unimi.it

Keywords: pig, carbon monoxide, endotoxic shock, oedema, VEGF

Abbreviations: CO, carbon monoxide; COHb, carboxyhemoglobin; HO, heme oxygenase; C_{rs}, respiratory system compliance; MPAP, pulmonary arterial pressure; EVLW, extravascular lung water; VEGF, endothelial vascular growth factor; R_{rs}, respiratory system resistances; LPS, lipopolysaccharides

INTRODUCTION

It is known that carbon monoxide (CO) is a toxic gas that rapidly leads to death by means of the action of carboxyhemoglobin (COHb). Recent work (Otterbein *et al*., 2000) has shown that low doses of CO induce anti-inflammatory and anti-apoptotic effects in endothelial cells. Moreover, in experimental animal models of cardiac transplant, CO administration was found to prolong the survival time of the graft (Bach *et al*., 1997; Brouard *et al*., 2000; Sato *et al*., 2001). CO is generated by the action of constitutive (HO-2) and inducible (HO-1) heme oxygenase, that are widespread in the organism. The expression of HO-1 is enhanced by cytokines and endotoxins and both HO-1 and CO suppress the pro-inflammatory response and promote the anti-inflammatory response of monocytes.

Because some cytokines may induce the vascular leakage that characterizes inflammatory and septic diseases, the aim of our work was to evaluate the possible protective effect of low doses of inhaled CO in an experimental model of swine endotoxic shock. In our model, we have studied the main functional and molecular parameters related to the onset of lung oedema, such as the compliance of the respiratory system (C_{rs}), the mean pulmonary arterial pressure (MPAP), the extravascular lung water (EVLW) and endothelial vascular growth factor (VEGF).

MATERIALS AND METHODS

The study was performed on eight Large White pigs, weighing 22.0 ± 2.69 kg (Mean ± d.s.). The animals, sedated with propionylpromazine hydrochloride 1% (0.05 ml/kg i.m.) and anaesthetised with thiopental sodium (bolus of 15 mg/kg, followed by an infusion of 9 mg/kg/h), were tracheostomised and mechanically ventilated with 100% O_2.

The respiratory flow and tidal volume were measured using a pneumotachograph (Fleisch n.2) inserted between the endotracheal tube and the ventilator, while tracheal pressure was measured by connecting a pressure transducer to a side port of the endotracheal cannula. A polyethylene catheter was inserted into the right femoral artery to monitor systemic arterial pressure and a Swan-Ganz catheter was introduced into the pulmonary artery to evaluate pulmonary arterial pressure (MPAP) and cardiac output. Intrathoracic blood and extravascular lung water volumes were evaluated using a thermodilution technique (PiCCO, 22G-5F; Pulsion Medical

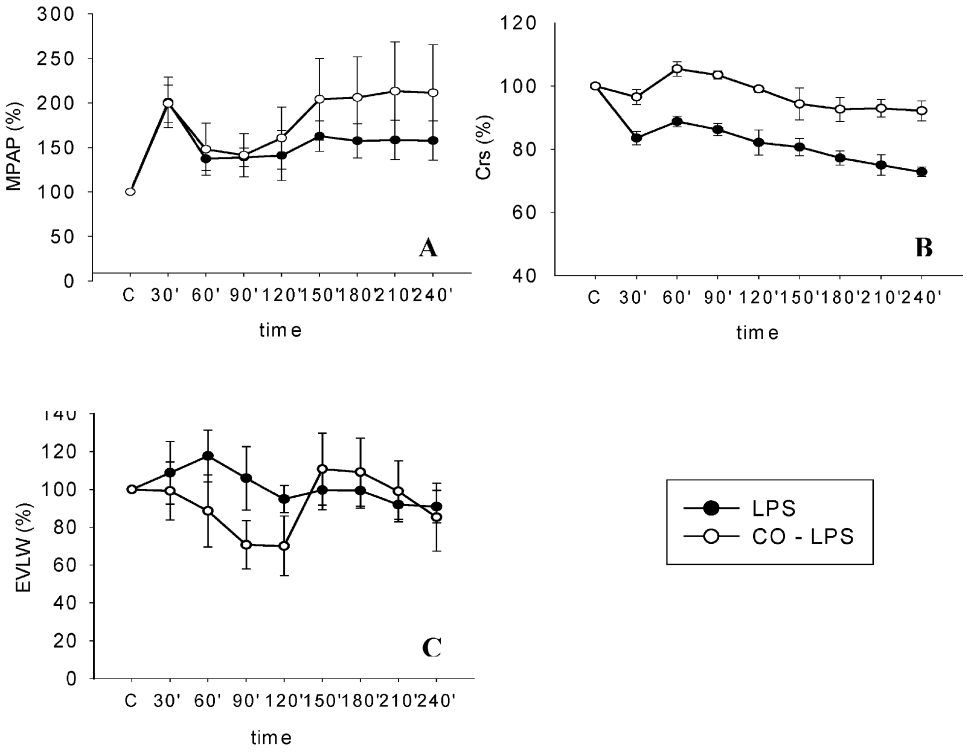


Figure 1. **A:** changes in mean pulmonary arterial pressure (MPAP); **B:** changes in compliance of the respiratory system (C_{rs}); **C:** changes in extravascular lung water (EVLW) for both groups of animals

System, Germany). All signals were recorded every 30 min on a multichannel pen recorder polygraph, while the compliance (C_{rs}) and the resistance (R_{rs}) of the respiratory system were calculated using standard formulas. Blood samples were collected for the ELISA test of VEGF. The animals of group 1 ($n = 4$) were treated with LPS of *E. coli* (40 μg/kg/h i.v. for 240 min) after 60 min of O_2 inhalation, while those of group 2 ($n = 4$) inhaled CO for 60 min (250 ppm in air), and then received LPS, as group 1.

At the end of the experiment, some samples of cardiac and pulmonary tissues were collected for HO-1 Western Blot analysis.

The statistical significance of the differences between baseline values and post-treatment conditions were evaluated using the T-Test for paired measurements. A p value of < 0.05 was considered to be statistically significant.

RESULTS AND CONCLUSIONS

Our results show that the administration of LPS induced a biphasic increase of mean pulmonary arterial pressure. The pre-treatment with carbon monoxide did not alter the early LPS-induced hypertensive response, while it did cause a greater increase of the MPAP in the last phase of experimental time.

Despite the strengthening of pulmonary hypertension, CO administration reduced the changes in C_{rs} induced by administration of LPS. These data, that indicate a protective mechanism exerted by CO on respiratory mechanics, are corroborated by the EVLW changes. Our results showed that, in animals treated with CO, the volume of extravascular lung water significantly decreased, particularly in the early phase of endotoxic shock. The plasma levels of VEGF in animals treated only with LPS were 416 ± 120 pg/ml, and those in animals pre-treated with carbon monoxide were between 0 and 15 pg/ml, confirming the hypothesis of a protective mechanism CO-induced on the altered capillary permeability due to LPS (Karmpaliotis *et al.*, 2002). In our experimental model, the expression of HO-1 in cardiac and lung tissues was not modified, confirming that activation of HO-1 requires a longer experimental period, as demonstrated in a previous "in vitro" study. Therefore, the results that we obtained in our hyper-acute model of endotoxic shock are due to inhaled CO, without any interference from neo-synthesized molecules in the tissue.

ACKNOWLEDGEMENTS

Work supported by MiPAF.

REFERENCES

Bach, F.H., Ferran, C., Hechenleitner, P., Mark, W., Koyamada, N., Miyatake, T., Winkler, H., Badrichani, A., Candinas, D. and Hancock, W.W., 1997. Accommodation of vascularized xenografts:

expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. *Nature Medicine*, **3**, 196–204.

Brouard, S., Otterbein, L.E., Anrather, J., Tobiasch, E., Bach, F.H., Choi, A.M. and Soares M.P. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis.2000. *Journal of Experimental Medicine*, **192**, 1015–1026.

Karmpaliotis, D., Kosmidou, I., Ingenito, E.P., Hong, K., Malhotra, A., Sunday, M.E. and Haley, K.J., 2002. Angiogenic growth factors in the pathophysiology of a murine model of acute lung injury. *American Journal of Physiology: Lung Cellular and Molecular Physiology*. **283**, L585-L595

Otterbein, L.E., Bach, F.H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., Davis, R.J., Flavell, R.A. and Choi, A.M., 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nature Medicine*, **6**, 422–428

Sato, K., Balla, J., Otterbein, L., Smith, R.N., Brouard, S., Lin, Y., Csizmadia, E., Sevigny, J., Robson, S.C., Vercellotti, G., Choi, A.M., Bach, F.H. and Soares M.P., 2001. Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *Journal of Immunology*, **166**, 4185–4194

Veterinary Research Communications, 28 (2004) 213–215

Ghrelin in the Gastroenteric Tract of Birds: Immunoreactivity Expression

S. Neglia*, N. Arcamone, V. Esposito and G. Gargiulo
Department of Biological Structures, Functions and Technology – Faculty of Veterinary Medicine, University of Naples "Federico II" – Italy
**Correspondence: Dipartimento di Strutture, Funzioni e Tecnologie Biologiche – Facoltà di Medicina Veterinaria, Università di Napoli "Federico II", Via Veterinaria, 1, I-80137 Napoli, Italy*
E-mail: sineglia@unina.it

Keywords: birds; food-intake; gastroenteric system; ghrelin

INTRODUCTION

Ghrelin is a 28 amino acid growth hormone-releasing peptide isolated from the rat stomach (Kojima *et al*., 1999). This molecule possesses a unique serine residue at the third position (Ser^3) that is modified by n-octanoid acid. Acylation is essential for ligand binding to the receptor GHS-R and subsequent ghrelin activity. In mammals ghrelin is expressed in a restricted hypothalamic area, the arcuate nucleus (Lu *et al*., 2002). This indicates that ghrelin may play a role in the regulation of feeding behavior and energy metabolism in the central nervous system (Date *et al*., 2000). Ghrelin is widely expressed in a variety of peripheral tissues, i.e. the stomach, intestine, placenta, kidney and pancreas (Kojima *et al*., 1999; Date *et al*., 2000; Mori *et al*., 2000; Dornonville de la Cour *et al*., 2001; Gualillo *et al*., 2001). Although little is known about ghrelin in non-mammalian vertebrates, ghrelin has been identified in the brain and stomach of a frog (*Rana esculenta*), showing the same acylation at the third aminoacidic residue as for mammals (Galas *et al*., 2002). Recently, Kaya *et al*. (2002) purified chicken ghrelin from the stomach of a chicken (*Gallus gallus*) and characterized its structure. Chicken ghrelin consists of 26 amino acids showing acylation at Ser^3 and the same sequence in the N-terminal region as for ghrelin from humans and rats. In the present study we have used antisera against rat ghrelin to investigate the distribution of ghrelin-like peptide in the gastroenteric system of the chicken.

MATERIALS AND METHODS

Three-month-old chickens *Gallus domesticus* of both sexes were used in this study. The gastrointestinal tract was sectioned in small samples obtained from the following segments: proventriculus, ventriculus, duodenum, jejunum, ileum and rectum (colon) (Baumel J). The tissues were immediately fixed in Bouin solution for 24 h, dehydrated

and embedded in paraffin. Sections of 6/7 μm in length were processed according to the peroxidase-antiperoxidase method, and incubated with a rabbit ghrelin against the N-terminal sequence of the rat ghrelin (ghrelin [1–11]) diluted 1:750. The specificity of the reaction was tested by omitting both primary or secondary antibodies, and utilizing primary antibody preabsorbed with an excess of antigen. The sections were examined using a Leitz Aristoplan microscope equipped with a Leica DC 300F digital camera.

RESULTS

Ghrelin immunoreactive cells (ghrelin-ic) were mainly found in the mucosal layer of the proventriculus and also in the small and large intestine. In the proventriculus ghrelin-ic were generally found at the base of glandular lobuli and in the basal zone of the plicae. Most of the ghrelin-ic were small and rounded in shape. These cells, intensely immunoreactive (ir) especially in the basal cytoplasm and named "closed-type cells", were not in contact with the lumen. A few ghrelin-ic, elongated in shape, showed an apical process that contacted the lumen and were the so-called "open-type cells". Ghrelin immunoreactivity decreases from the pilorus toward the enteric distal tracts. In the duodenum in particular the two types of ghrelin-ic, "closed" and "open" cells, were still observed along villi intestinales. Ghrelin-ic were less numerous in the jejunum and ileum, and were generally of the "closed" type. In the colon rare triangular-shaped "closed" ghrelin-ic were detected.

DISCUSSION

In this study we have clearly demonstrated that ghrelin producing cells exist in the chicken gastrointestinal tract. The distribution pattern and morphological characteristics of ghrelin-ic suggest that they correspond to endocrine type cells. However, further investigations are required in order to identify the specific cell types according to the current classification (Solcia *et al*., 2000). In the frog stomach ghrelin endocrine cells have been observed in the mucosa and in pit-associated glands (Galas *et al*., 2002). In the rat and human stomach ghrelin-producing endocrine cells have been identified as X/A like cells of the oxyntic mucosa (Kojima *et al*., 1999; Date *et al*., 2000). These results indicate that chicken ghrelin is also evolutionally conserved both in its distribution and in its aminoacidic sequence.

REFERENCES

Date, Y., Kojima, M., Hosoda, H., Sawaguchi, A., Mondal, M.S., Suganuma, T., Matsukura, S., Kangawa, K. and Nakazato, M., 2000. Ghrelin, a novel ghrowth hormone-releasing acylated peptide is

synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology*, **141**, 4255–4261

Dornonville de la Cour, C., Bjorkqvist, M., Sandvik, A.K., Bakke, I., Zhao, C.M. and Chen, D., 2001. A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. *Regulatory Peptides*, **99**, 141–150

Galas, L., Chartrel, N., Kojima, M., Kangawa, K. and Vaudry, H., 2002. Immunoistochemical localization and biochemical characterization of ghrelin in the brain and stomach of the frog *Rana esculenta*. *Journal of Comparative Neurology*, **450**, 34–44

Gualillo, O., Caminos, J.E., Blanco, M., Garcia-Caballero, T., Kojima, M., Kangawa, K., Dieguez, C. and Casanueva, F., 2001. Ghrelin, a novel placental-derived hormone. *Endocrinology*, **142**, 788–794

Horvarth, T.L., Diano, S., Sotonyi, P., Heiman, M. and Tschop, M., 2001. Ghrelin and the regulation of energy homeostasis: a hypothalamic perspective. *Endocrinology*, **142**, 4163–4169

Hosoda, H., Kojima, K., Matsuo, H. and Kangawa, K., 2000. Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor. *Journal of Biological Chemistry*, **275**, 1195–2000

Kaiya, H., Van der Geyten, S., Kojima, M., Hosoda, H., Kitajima, Y. and Matsumoto, M., 2002. Chicken ghrelin: purification, cDNA cloning, and biological activity. *Endocrinology*, **143**, 3454–3463

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H. and Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, **402**, 656–660

Lu, S., Guan, J.L., Wang, Q.P., Uchara, R., Yamada, S., Goto, N., Date, Y., Nakazato, M., Kojima, M., Kangawa, K. and Shioda, S., 2002. Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. *Neuroscience Letters*, **321**, 157–160

Mori, K., Yoshimoto, A., Takaya, K., Hosoda, K., Ariyasu, H., Yahata, K., Mukoyama, M., Sugawara, A., Hosoda, H., Kojima, A., Kangawa, K. and Nakao, K., 2000. Kidney produces a novel acylated peptide, ghrelin. *FEBS letters*, **486**, 213–216

Solcia, E., Rindi, G., Buffa, R., Fiocca, R. and Capella, C., 2000. Gastric endocrine cells: types, function and growth. *Regulatory Peptides*, **93**, 31–35

Veterinary Research Communications, 28 (2004) 217–219

Effects of Acetyl-salicylate Used in Post-calving of Dairy Cows

G. Bertoni*, E. Trevisi and F. Piccioli-Cappelli
Institute of Zootechnics, Faculty of Agriculture, Università Cattolica del Sacro Cuore, Piacenza, Italy
**Correspondence: Istituto di Zootecnica, Facoltà di Agraria, Università Cattolica del Sacro Cuore, via Emilia Parmense 84, 29100 Piacenza, Italy*
E-mail: giuseppe.bertoni@unicatt.it

Keywords: acetyl-salicylate, dairy cows, milk yield, fertility, blood indices

INTRODUCTION

High yielding dairy cows are particularly vulnerable, during the transition period, to any event able to stimulate the immune system (viruses, bacteria, many kind of stress, endotoxins, trauma, etc.). Pro-inflammatory cytokines (i.e. IL-1, IL-6, TNFα), mainly released from monocyte-macrophage cells, are the common mediators of these events. Cytokines are able to activate other cellules like those of hypothalamus, liver, reproductive organs etc. (Elsasser *et al*., 1997). As previously stressed (Bertoni *et al*., 2000; Trevisi *et al*., 2002), the consequent endocrine-metabolic changes can cause marked negative effects: anorexia, lipomobilization, reduction of milk yield and reproductive traits. Therefore, the modulation of cytokine release and/or the inhibition of some related effects could represent a noticeable target in the transition period. The aim of our study concerned the evaluation of the effects of a preventive administration of a well-known anti-inflammatory drug (acetyl-salicylate, able to inhibit both cyclo-oxygenases – COX-1 and COX-2 – the key enzymes of inflammatory response) in post-calving dairy cows.

MATERIALS AND METHODS

The research was carried out in a commercial herd where dry cows were fed with 3 kg of alfalfa hay and grass hay *ad libitum*, while lactating cows received TMR *ad libitum* (28.3% maize-silage, 21.2% grass hay, 3.8% alfalfa hay, 46.7% concentrate all on dry matter basis). Twenty-two pluriparous cows were allocated in two homogeneous groups, CTR and LAS, according to their calving number, BCS and time from calving. LAS received daily i.m. injections of lysine acetyl-salicylate (Gellini Farmaceutici, S.p.a., LT, Italy) in the first 5 days of lactation (DIM): 15 g/d in the first 3 DIM and 7.5 g/d in the following 2 days. The CTR group was used as a control. The milk yield (3, 7, 10, 14, 21, 28, 42, 56, 70, 90, 120 DIM), BCS (−7, 14, 28, 42, 56 DIM), metabolic profile (3, 7, 10, 14, 21, 28, 46, 56 DIM), disease occurrence and fertility were monitored. In addition, representative feed samples were collected for proximate analysis

and the evaluation of rations. The statistical analysis was performed by SAS™ (8 TS M0 version) MIXED procedure, using cow within treatment, DIM, treatment and DIM-treatment interaction as factors. The lactation curve of each cow was estimated according to Wood equation by Proc. NLIN of SAS™.

RESULTS

During the trial, the diet composition did not change either in dry (0.70 UFL/kg, d.m., 11.6% CP d.m.) or in lactating groups (0.92 UFL/kg, d.m., 15.1% CP d.m.). Dry matter intake (DMI) was also acceptable in dry and lactation periods, 11.3 and 22.9 kg/d respectively; but fresh cows showed an energy (–6%) and a protein (–15%) deficiency, according to their milk yield level. LAS cows showed: a higher milk yield ($p < 0.10$ at the peak; Fig. 1); a more marked (−1.08 vs −0.92 score of CTR) and more prolonged reduction of BCS; a lower frequency of anorexia at the beginning of lactation (10 vs 45% of CTR). Conversely, LAS was affected by higher frequency of metritis (30 vs 18% of CTR), although the reproductive troubles that were observed during the trial were similar (about 50%) in the two groups. Moreover, LAS showed better fertility traits: more cows pregnant after 1st insemination (50 vs 27%), less services per conception (2.11 vs 2.33 n°), a slight reduction of open days (126 vs 133 d) and fewer cows culled for infertility (1 vs 2).

During the 1st week of lactation, LAS showed a lower level of glucose ($p < 0.05$ at 3 DIM) and higher levels of NEFA and GOT in comparison to CTR, suggesting a more marked energy deficiency explained by the higher milk production. Nevertheless, the pattern of changes of plasma βOHB did not differ between groups, while urea reached higher levels (but within reference interval) in LAS. Furthermore, LAS showed more favourable indices of inflammatory status: higher Zn ($p < 0.05$ at 7 DIM), lower acute phase proteins (haptoglobin and particularly ceruloplasmin, as shown in Fig. 1), total proteins and globulins ($p < 0.05$). The less severe inflammatory

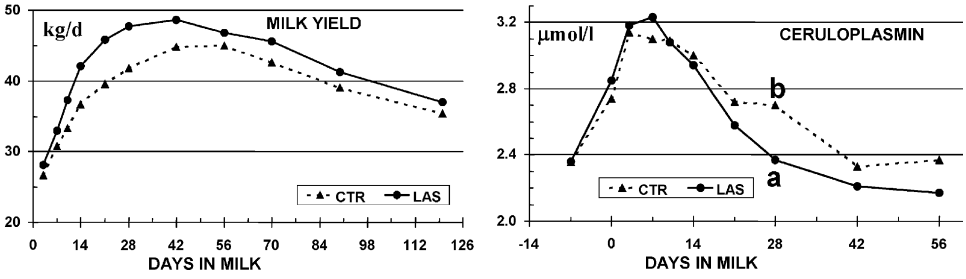


Figure 1. Behaviour of milk yield and plasma ceruloplasmin in dairy cows treated (LAS) or not (CTR) with lysine acetyl-salicylate in post-calving. Different letters showed a significant difference between groups (a,b: $p < 0.05$)

processes observed in LAS had considerably improved the levels of proteins (sometimes in a significant manner) of common liver synthesis: albumin, lipoproteins (total cholesterol) and retinol-binding protein (vitamin A).

DISCUSSION

The treatment with acetyl-salicylate seems to minimize the undesired consequences of the inflammatory events around calving, which occurred also in treated cows, as confirmed by the increase of acute phase proteins. The positive effects of LAS treatments could also be seen in the quicker increase of DMI that was suggested by the lower frequency of anorexia and the similar levels of βOHB, despite a more pronounced energy deficiency (confirmed by the more marked reduction of BCS). Moreover, the higher DMI of LAS also seems supported by the higher plasma urea level, mainly related to the total protein intake when diet composition is unchanged. The better health status (i.e. more favourable indices of inflammation) and perhaps the higher feed intake of LAS allowed a prompt recovery of common metabolic conditions, a better liver activity, a rise in milk yield and an improvement in fertility. In conclusion, the use of molecules able to modulate the eicosanoids after calving seems promising, but further research is required.

ACKNOWLEDGEMENTS

This research was supported by P.F. R.A.I.Z., publication n° RZ 288.

REFERENCES

Bertoni, G., Calamari, L. and Trevisi, E., 2000. Alterazioni della funzionalità epatica nel puerperio e conseguenze sulla fertilità della bovina. In G. Enne, G. Rossi (eds), *La riproduzione in zootecnia*, vol. III, 195–208

Elsasser, T.H., Kahl, S., Steele, C. and Rusmey, T.S., 1997. Nutritional modulation of somatotropic axis-cytokine relationships in cattle: a brief review. *Comparative Biochemistry Physiology*, **116**A(3), 209–221

Trevisi, E., Han X.-T., Piccioli-Cappelli, F. and Bertoni, G., 2002. Dry matter intake reduction before calving in dairy cows: relationship with immune system and metabolism conditions. *53rd Meeting European Association Animal Production*, Cairo, Egypt, Sept. 1–4, 54

Veterinary Research Communications, 28 (2004) 221–224

Use of Two Different Energy-value Diets on Sarda Ewes During Late Pregnancy and Suckling

F. Bovera, R. Schettini, M.I. Cutrignelli, S. Calabrò, F. Infascelli and V. Piccolo
Dipartimento di Scienze Zootecniche e Ispezione degli alimenti, University of Naples "Federico II"
Correspondence, Dipartimento di Scienze Zootecniche e Ispezione degli alimenti, via F. Delpino, 1, 80137, Naples, Italy
E-mail: bovera@unina.it

Keywords: Lambs, non-structural carbohydrates, Sarda ewe

INTRODUCTION

The last month of pregnancy and suckling are two critical periods in the sheep production cycle. Moreover, in both cases, the nutritive requirements increase while the dry matter (DM) intake is not adequate. Therefore, energy deficits may well occur with negative consequences on the body weight of lambs at birth and on subsequent milk production. Thus the aim of our paper was to study the effect of two diets differing in energy value on the productive performance of Sarda ewes during the last month of pregnancy and suckling.

MATERIALS AND METHODS

The study was carried out on a Sarda sheep farm in the province of Caserta in which the animals were bred in the stall system. The study concerned 30 pregnant sheep, for which single births had been diagnosed by echograph. One month before lambing, they were divided into two groups, whose diets consisted of (% on dry matter): maize silage (35), cut oat hay (30) and concentrate (35), the latter two differing for the two groups. Group A was fed a commercial concentrate and group B received a mix of the concentrate (14), maize (13.4) and soybean meal (7.6) in order to diversify the NSC contents of the two diets, maintaining the same percentage of crude protein. Table I shows the chemical characteristics of the diets (ASPA, 1980; Martillotti *et al*., 1987). The nutritive value of the diets was estimated following INRA (1988) indications.

Under the rationing scheme, different quantities of DM according to body weight (BW) were administered twice daily as TMR (2% BW at the 5th gestation month and colostral period, 3.7% from the 5th to 7th day; 4.5% from the 8th to 14th day and 5% from the 15th). After lambing, due to complications, two sheep from group A and three from group B were eliminated.

TABLE I
Chemical-nutritional characteristics of the two diets

	% DM	
	A	B
Crude protein	14.06	14.05
Crude fibre	18.31	18.16
Ether extract	2.77	2.51
Ash	8.86	7.68
NDF	40.74	38.59
ADF	26.06	24.49
ADL	2.87	3.10
Starch	20.74	26.10
NSC	35.94	39.92
MUF/kg DM	0.82	0.90

TABLE II
Weight at birth (WB), at sale (WS), weight gain (WG) and milk consumed by lambs (MC) as a function of diet and sex

	WB, kg	WS, kg	WG, g/d	MC, g/d
		Diet effect (DF = 1)		
A	3.56	10.32	217.1	1370.9
B	3.47	11.52	253.5	1491.9
		Sex effect (DF = 1)		
F	3.05	9.66	201.7	1289.5
M	4.25	12.24	270.2	1580.1
		Significant		
D[a]	NS	*	NS	NS
S[b]	**	**	**	**
D × S[c]	NS	NS	NS	NS
		Variance of error (DF = 47)		
	0.24	1.43	2407.8	52959.5

a: diet effect; b: sex effect; c: interaction diet × sex.

The male/female ratio between lambs born was 54:46 in group A and 50:50 in group B. The lambs were weighed at birth and at sale (30 days old) in order to calculate their weight gain during suckling. With such measurements we were able to estimate the average amount of milk intake from lambs during suckling using the equation developed by Poujardieu (1969). All the results obtained were processed

using the GLM procedure of SAS (2000) following the model: $y_{ijk} = \mu + \alpha_i + \alpha_j + \alpha\beta_{ij} + \varepsilon_{ijk}$, where y_{ijk} is the single observation, α_i is the diet effect ($i = 1, 2$), $\alpha_j =$ is the sex effect ($j =$ male or female), $\alpha\beta_{ij}$ is the interaction between diet and sex, and ε_{ijk} is the error.

RESULTS

Table II shows the results relative to: weight at birth and at sale, weight gain (WG) and the milk consumed by lambs (MC) according to mothers' diet and sex. The different NSC content of the diets induced statistically significant differences ($p < 0.05$) only for weight at sale. Interestingly, WG and MC were higher for the group that received the diet with the highest nutritive value. The results, in agreement with those of Cannas (2001), could be due to the high level of somatotrope hormone (SH) present in the blood during the first period of lactation: this would also direct energy use (and hence NSC use) towards milk production instead of the deposition of body fat (Peel *et al*., 1987). Regarding the differences between the sexes, the males showed higher values ($p < 0.01$) for all the examined parameters. Diet × sex interaction was never found to be statistically significant.

CONCLUSIONS

The results obtained show that use of the diet with a higher NSC content could improve the productive performance of primiparous Sarda ewes during the last month of pregnancy and suckling. The absence of statistically significant differences between body weight and milk consumed by lambs could be justified by the high variability of the results obtained.

ACKNOWLEDGEMENTS

We would like to thank Maria Ferrara for her technical collaboration, and the "Mastrogiovanni Giorgina" farm of Pignataro Maggiore (Caserta) for helping us to carry out the trial.

REFERENCES

ASPA, 1980. Commissione valutazione alimenti. Valutazione degli alimenti di interesse zootecnico. Analisi chimica. *Zootecnica e Nutrizione Animale*, **6**, 19–34

Cannas A., 2001. Tecniche di alimentazione delle pecore e stima del valore energetico e proteico degli alimenti. In: *L'alimentazione degli ovini da latte*. Avenue Media, Bologna, 111–165

INRA, 1988. *Alimentation des bovins, ovins et caprins*. Institut National de la Recherche Agronomique, Paris

Martillotti, F., Antongiovanni, M., Rizzi, L., Santi, E. and Bittante, G., 1987. *Metodi di analisi per la valutazione degli alimenti di impiego zootecnico*. Edizioni IPRA, CNR, Roma

Peel, C.J. and Bauman, D.E., 1987. Somatotropin and lactation. *Journal of Dairy Science*, **70**, 474–486

Poujardieu B., 1969. Recherche d'une méthode d'estimation de la production laitière des femelles ovines et bovines pendant la phase d'allaitement. *Annales de Zootechnie*, **18**(3), 299–315

SAS, 2000. SAS/STAT® Software: Changes and Enhancements through Release 8.1. SAS Institute Inc., Cary, NC

Veterinary Research Communications, 28 (2004) 225–227

Sensory Evaluation of Sea Bass (*Dicentrarchus labrax* L.) Fed Two Diets Differing in Fat Content

R.M. Lopparelli[1]*, S. Segato[2], A. Corato[2], L. Fasolato[2] and I. Andrighetto[2]
[1] *Department of Public Health, Comparative Patology and Veterinary Hygiene;*
[2] *Department of Animal Science, Padova University, Viale dell'Università 16, 35020 Legnaro (PD), Italy*
* *Correspondence: Centro per la Sicurezza Igienico Sanitaria degli Alimenti, via S. Gaetano, 36016 Thiene (VI), Italy*
E-mail: rosa.lopparelli@unipd.it

Keywords: European sea bass, mariculture, dietary fat, sensory evaluation

Abbreviations: BW, body weight; L*, lightness; a*, redness; b*, yellowness

INTRODUCTION

Feeding of marine fish widely uses the strategy which consists of the partial substitution of crude protein with lipids (protein-sparing effect). However, high dietary lipid content modifies whole body chemical composition, resulting in a higher adipogenetic deposition which influences the organoleptic quality of fish products (Segato *et al*., 2003a). This influence on quality can be assessed by sensory analyses such as a discriminative taste panel. The aim of this study was to evaluate the sensory quality of fish in relation to dietary fat content on organoleptic parameters of European sea bass reared in mariculture.

MATERIALS AND METHODS

European sea bass (initial body weight: 304 ± 30 g) were reared in two floating cages (density: 9 kg/m^3) placed in the Gulf of Trieste. During summer and autumn of 2001 (97 experimental days) fish were fed (1% BW) two isoproteic diets with different crude fat level (Low vs. High). Proximate composition (AOAC, 2000) and gross energy of the two experimental diets are reported in Table I.

At the end of the trial, 30 fish (final body weight: 412 ± 26 vs 407 ± 22 g) from each experimental group were caught and killed on ice. After 48 h conservation at $2 \pm 1^{\circ}$C, discriminative sensory analysis was carried out on fish ($n = 8$) by 10 trained tasters. Gutted trunks were wrapped in tinfoil and cooked in an electric oven at 165°C until the fillet core temperature reached 60°C. Jury evaluation considered the following attributes: trunk compactness, exudation and skin detachment; fillet (white epiaxial muscle) odour, lightness, tenderness, juiciness, marine and salty flavour and taste. The

TABLE I
Proximate composition (% w.w.) and gross energy (MJ/kg w.w.) of the experimental diets

Diet	Crude protein	Ether extract	N-Free extract	Ash	Gross energy
Low	42.9 ± 1.7	19.4 ± 1.1	22.4 ± 1.6	8.5 ± 0.4	21.4 ± 0.4
High	43.2 ± 0.2	24.6 ± 0.5	16.0 ± 1.0	9.2 ± 0.2	22.5 ± 0.2

Means ± SD.

score was expressed on a 9-point scale (1 = low ... 9 = high) using the attributes schedule in Table II. For tasting each judge considered the same dorsal muscular portion, avoiding intermuscular adipose deposits. Data were submitted to one-way ANOVA according to a mono-experimental design (diet effect) by PROC GLM of SAS (1999).

RESULTS AND DISCUSSION

Trunk compactness and significant visible exudation were lower for fish fed with the High diet (Table II). This result is probably due to the higher fillet fat content observed in High diet fed fish (7.7% vs. 8.9%; $p < 0.1$ – data not tabulated). The fillet structure is influenced by intra- and perimuscular fat content and its location which modify texture and water hold capacity (Torrissen *et al.*, 2001). The higher exudative quantity observed in the fish fed the Low diet trunk was probably due to moisture losses caused by more intensive protein denaturation during heating treatment. On

TABLE II

	Dietary lipid content			
Attribute (discriminant)	Low	High	*p*	SEM
Trunk compactness (compact)	6.2	5.5	†	0.3
Trunk exudation (exudates)	5.8	3.7	*	0.6
Skin detachment (detachable)	7.0	6.2	ns	0.9
Fillet odour (marine)	6.5	6.4	ns	0.6
Fillet lightness (light)	6.2	6.0	ns	0.2
Fillet tenderness (tender)	5.6	6.5	*	0.2
Fillet juiciness (juicy)	5.4	5.9	†	0.1
Fillet marine flavour (marine)	5.1	5.2	ns	0.6
Fillet salty flavour (salty)	4.8	4.0	ns	0.3
Fillet taste (agreeable)	6.5	6.2	ns	0.1

† $p < 0.1$; * $p < 0.05$.

the contrary, fish fed the higher fat content diet showed higher values of fillet juiciness and tenderness. These quality traits also are dependent on the higher level of muscular fat. With regard to juiciness, suitable marbling limits cellular liquid losses during heating and induces chewing salivation. Lipids also limit the cohesion between muscular fibres and between muscular and collagen fibres, reducing firmness of the cooked flesh (Segato *et al*., 2003b; Torrissen, *ibidem*). Data of sensory tenderness were confirmed by maximum shear force determination (7.8 vs. 6.0 N; $p < 005$ – data not tabulated). Panellists did not detect any differences in fillet lightness while instrumental measurements using the Hunter-L*a*b* method showed significant differences (L*:77.6 vs. 81.4; $p < 0.01$ – data not tabulated). Some sensory parameters (i.e. colour) showed thresholds which are not perceptible even by trained panellists. Organoleptic traits such as odour, flavour and taste were not influenced by diet any factors. Considering the dietary fat range tested (18–25%), this result shows that differences detected by smell and taste of cooked products could mainly be related to the properties of the raw materials used in the fattening diet rather than to the level of crude fat administered.

In conclusion, feeding a high-fat diet to mariculture European sea bass leads to higher adipogenesis both as lipidic deposits and as intramuscular fat; flesh from this rearing condition is characterized by lower cooking losses and higher juiciness and tenderness.

ACKNOWLEDGEMENTS

The research was supported by MIPAF – Direzione Generale Pesca e Acquacoltura.

REFERENCES

AOAC, 2000. *Official Methods of Analysis*, 17th Ed. (AOAC International, Gaithersburg, VA)

Johansson, L., 2001. Eating quality of farmed rainbow trout (*Oncorhynchus mykiss*). In: S.C. Kestin and P.D. Warriss (eds), *Farmed Fish Quality* (Fishing New Books – Blackwell Science, Oxford), 76–88

SAS, 1999. SAS *User's Guide: Statistics* Version 6.03 (SAS Institute, Cary, NC)

Segato, S., Lopparelli, R.M., Borgoni, N., Zanella, L., Corato, A. and Andrighetto, I., 2003a. Effect of dietary crude fat to NFE ratio on growth, feed efficiency and quality traits in juvenile shi drum (*Umbrina cirrosa*). Cahiers Options Méditerrannéennes (in press)

Segato, S., Lopparelli, R.M., Francescon, A., Barbaro, A., Trisolini, R. and Andrighetto, I., 2003b. Effetto della ploidia sulle caratteristiche qualitative di ombrina (*Umbrina cirrosa*, L). *Biologia Marina Mediterranea*, **10**(2) (in press)

Torrissen, O.J., Sigurgisladottir, S. and Slinde, E., 2001. Texture and technological properties of fish. In: S.C. Kestin and P.D. Warriss (eds), *Farmed Fish Quality* (Fishing New Books – Blackwell Science, Oxford), 31–41

Veterinary Research Communications, 28 (2004) 229–232

Survey of Ochratoxin A in Cereals from Puglia and Basilicata

M. Muscarella[1]*, C. Palermo[2], T. Rotunno[2], V. Quaranta[1] and P. D'Antini[1]
[1] *Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Foggia, Italy;*
[2] *Dipartimento di Scienze Agro-ambientali, Chimica e Difesa vegetale – Facoltà di Agraria – Università degli Studi di Foggia, Italy*
* *Correspondence: Dipartimento di Chimica. Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Via Manfredonia, 20, 71100 Foggia, Italy*
E-mail: m.muscarella@izsfg.it

Keywords: cereals, mycotoxin, ochratoxin A

Abbreviations: O.A, ochratoxin A; BEN, Balkan endemic nephropathy; IARC, International Agency for Research in Cancer; EC, European Community

INTRODUCTION

Ochratoxin A (O.A), a secondary metabolite with teratogenic, hepatotoxic, carcinogenic and nephrotoxic activity in many animal species, including human beings (Hohler, 1998), is mainly produced by *Aspergillus ochraceus* and *Penicillium viridicatum.* Being ubiquitous, these moulds can easily contaminate foodstuffs. O.A is predominantly found in cereal grains, cereal products, legumes, oilseed, coffee beans and feed. Moreover, O.A has been identified in the tissue and organs of animals (pigs, chicken) fed contaminated feed. The presence of O.A has also been reported in human milk. The IARC (International Agency for Research in Cancer) has classified O.A as Group 2B, a possible human carcinogen (IARC, 1993). In several areas of Eastern Europe, where chronic exposure to O.A occurs, involvement of this mycotoxin in the cancer aetiology of the urinary system, and in kidney pathologies typical of Balkan Endemic Nephropathy (BEN) has been suspected. Studies into the correlation between O.A and BEN (Puntaric *et al*., 2001) have shown higher O.A contamination levels in cereals from endemic areas with respect to cereals from non-endemic areas. The high risk for Public Health represented by such a mycotoxin and the insufficient epidemiological data relevant to Italian regions, which neighbour the Balkan area, suggested to us the present survey aimed at evaluation of overall status of O.A contamination levels in some cereals produced in Puglia and Basilicata, which are regions where the economy is primarily based on agriculture.

MATERIALS AND METHODS

260 cereal samples (95 durum wheat, 80 maize, and 85 barley) from Puglia and Basilicata were examined. Just before analysis, each sample was finely ground with a

Cemotec 1090 mill (Tecator) to a particle size of < 1 mm, 25 g of homogenized sample was taken for analysis, using the procedure described by Nesheim (Nesheim *et al*., 1992).

O.A was extracted with chloroform-aqueous phosphoric acid, and isolated by liquid-liquid partitioning into an aqueous bicarbonate solution. The extract was transferred to a C_{18} solid-phase extraction (SPE) column, and O.A was eluted with ethyl acetate–methanol–acetic acid (95/5/0.5). O.A was identified by reversed-phase Liquid Chromatography (LC) and quantified by fluorescence detection. The performance of this method has been evaluated and described by Palermo (Palermo *et al*., 2002) in a previous review.

Apparatus: The chromatographic system used was a Waters Model 600 E (Waters Milford, MA, USA) equipped with a Waters Model 579 fluorescence detector and a Waters Model 5200 integrator-recorder.

Reagents: Ethyl acetate, chloroform, benzene, methanol and water were HPLC grade, and acetic acid, sodium bicarbonate and phosphoric acid were ACS grade (J.T.Baker, Koeln, Germany). Celite 545 (Merck, Darmstadt, Germany), SPE-Isolute-C_{18} microcolumns (Intern.Sorbent Techn., Mid Glamorgan, U.K.). Standard O.A was purchased from Sigma (Sigma Chem. Co., St Louis, Missouri, USA).

Chromatographic conditions: The LC column used was a Supelcosil C_{18} reversed-phase packing, 4.6×250 mm, 5 μm film thickness (Supelco, Bellefonte, PA, USA). The mobile phase was a water-acetonitrile-acetic acid (99/99/2) mixture and the flow rate was 0.8 ml/min. The temperature of the column was 30°C and the injection loop was 20 μl. The fluorescence detector was regulated at 330 nm for excitation and 470 nm for emission wavelengths.

RESULTS AND DISCUSSION

The results on the O.A levels in various cereal samples are reported in Table I. O.A was detected ($c >$ LOD) in 20.4% of examined samples. Only one maize sample from Trinitapoli (Foggia) had an O.A content (5.2 ng/g) above the maximum tolerance limit for O.A in cereals (5.0 ng/g), recently fixed by the European Community Regulation no. 472/2002.

The highest frequency of contamination was in the range of LOD – 0.9 ng/g for 13.5% of examined samples, while only 6.5% had an O.A value between 1.0 to 4.9 ng/g. The highest mean level of contamination (30.0%) was found for maize.

Overall, the obtained data are reassuring. In fact, only 1.3% of samples were contaminated with a value higher than the maximum limit of tolerance of Ochratoxin A in cereals as fixed by the European Community.

The distribution of the O.A positive samples, according to the areas where the sampled cereals were produced, is reported in Table II. The presence of O.A is homogeneous, however it does not represent a relevant health risk.

The present survey has revealed a very low percentage (1.3%) of cereal samples

TABLE I
Content of Ochratoxin A in cereal samples from Puglia and Basilicata

Cereals	No. samples	> LOD[a] *n* (%)	< LOD *n* (%)	LOD – 0.9 ng/g n (%)	1.0–4.9 ng/g *n* (%)	> 5.0 ng/g *n* (%)	Range ng/g	Average $\pm\sigma$
Durum wheat	95	15 (15.8)	80 (84.2)	13 (13.7)	2 (2.1)	—	0.2–3.9	0.8–0.3
Maize	80	24 (30.0)	56 (70.0)	15 (18.7)	8 (10.0)	1 (1.3)	0.1–5.2	1.8–1.5
Barley	85	14 (16.4)	71 (83.5)	7 (8.2)	7 (8.2)	—	0.1–3.9	2.0–1.0
Totals	260	53 (20.4)	207 (79.6)	35 (13.5)	17 (6.5)	1 (0.5)	0.1–5.2	1.5–1.2

[a] LOD = 0.05 ng/g.

TABLE II
Distribution of O.A positive ($c >$ LOD) cereal samples in different areas of Puglia and Basilicata

Origin	Durham wheat			Maize			Barley		
	No. samples	$c >$ LOD *n* (%)	$\bar{x}$	No. samples	$c >$ LOD *n* (%)	$\bar{x}$	No. samples	$c >$ LOD *n* (%)	$\bar{x}$
Cerignola	10	—	—	10	2 (20)	0.6	10	1 (10)	1.3
Foggia	20	—	—	20	6 (30)	1.6	13	2 (15)	0.7
Lucera	10	—	—	20	2 (10)	0.6	20	—	—
Manfredonia	10	3 (30)	0.9	5	3 (30)	0.9	10	1 (10)	2.2
Potenza	25	9 (36)	0.7	10	5 (50)	0.3	15	6 (24)	0.2
Trinitapoli	10	—	—	5	3 (30)	4.0	10	2 (20)	3.4

from Puglia and Basilicata with an O.A concentration above the EC legal limit. This is a reassuring finding on the overall status of the O.A contamination level in the cereals produced in these regions of Italy. O.A contamination was in the range of 1.0 to 4.9 ng/g in 6.5% of examined samples, a fact that indicates that the surveillance of O.A in cereals and other matrices should be continued, in compliance with the EC programs on food quality assurance.

REFERENCES

E.C.R. 2002 (European Communities Regulation no. 472/2002, of March 12, 2002). *The Official Journal of the European Communities*, L75/18, March 16, 2002

Hohler, D., 1998. Ochratoxin A in food and feed: occurrence, legislation and mode of action. *Z. Ernahrungswiss*, **37**, 2–8

IARC (International Agency for Research on Cancer) 1993. In *IARC Monographs on the Evaluation of*

Carcinogenic Risks to Humans: Some Naturally Occurring Substances; Food Items and Costituents, Heterocyclic Aromatic Amines and Mycotoxins, **56**, 489, IARC (Ed.), Genere

Nesheim, S., Stack, M.E., Trucksess, M.W. and Eppley R.M., 1992. Rapid solvent efficient method for liquid-chromatographic determination of Ochratoxin A in corn, barley, and kidney: collaborative study. *Journal of Association of Official Analytical Chemists*, **75**, 481–487

Palermo, D., Pietrobono, P., Palermo, C. and Rotunno, T., 2002. Occurrence of Ochratoxin A in cereals from Puglia (Italy). *Italian Journal of Food Science*, **4**(14), 447–453

Puntaric, D., Bosnir, J., Smit, Z., Skes, I. and Baklaic, Z., 2001. Ochratoxin A in corn and wheat: geographical association with endemic nephropathy. *Croatian Medical Journal*, **42**, 175–179

Veterinary Research Communications, 28 (2004) 233–236

First Pregnancies Carried to Term After Transfer of Vitrified Buffalo Embryos Entirely Produced *in vitro*

G. Neglia*, B. Gasparrini, V. Caracciolo di Brienza, R. Di Palo and L. Zicarelli
DISCIZIA, Faculty of Veterinary Medicine, "Federico II" University, Via F. Delpino, 1, Naples, Italy
**Correspondence: DISCIZIA – Sez. B. Ferrara – Faculty of Veterinary Medicine – Via F. Delpino, 1, 80137, Naples, Italy.*
E-mail: neglia@unina.it

Keywords: ovum pick-up, vitrification, embryo transfer, buffalo

INTRODUCTION

The application of Ovum Pick-up (OPU) technology, together with multistep embryo production *in vitro* (IVEP), represents a valid procedure for the recovery of oocytes from live donors and the attainment of a large number of embryos (Looney *et al*., 1994; Galli *et al*., 2001). In fact, collection of oocytes by means of ultrasound guided follicular aspiration is routinely performed with success in large prepubescent and adult ruminant species (Galli *et al*., 2001). In the buffalo species, because of their low response to superovulation treatments (Karaivanov, 1986), OPU technology, together with an improvement in the multistep process of *in vitro* embryo production, seems to be the best way to enhance genetic progression through the maternal lineage (Galli *et al*., 2001; Neglia *et al*., 2003). However, *in vitro* embryo production in buffalo has always been associated with a lower efficiency as compared to the bovine (Totey *et al*., 1992), although recently some encouraging results have been reported both from slaughterhouse recovered oocytes (Gasparrini *et al*., 2000), and from OPU-derived oocytes (Neglia *et al*., 2003). Obviously, in order to optimize the procedure, an efficient method for embryo freezing should be developed. In fact, until now the only pregnancies recorded for the buffalo species were obtained from the transfer of fresh embryos produced *in vitro*. In 1991 Madan *et al.* (Madan *et al*., 1991) reported the birth of the first buffalo calf after the transfer of a fresh blastocyst obtained through *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and in co-culture with oviductal cells (IVC). Four other calves were produced in India in 1994, by transfer of fresh blastocysts obtained *in vitro* (Madan *et al*., 1994), using the same method. In 1997, Boni *et al.* (Boni *et al*., 1997), reported two pregnancies obtained from the transfer of two fresh morulae, produced after OPU/IVEP procedures. Only three calves have been produced from frozen blastocysts (Galli *et al*., 1998); in this case zygotes (2 days after IVF) were transferred into ligated sheep oviduct before freezing. The possibility of cryopreserving buffalo embryos by vitrification with good efficiency in terms of *in vitro* survival rate, has recently been reported (Neglia *et al*., 2001). On

the basis of these results a one month trial was carried out on a buffalo farm in order to verify the efficiency of this freezing method, by transferring vitrified and fresh embryos produced using OPU-IVEP technology.

MATERIALS AND METHODS

In March 2002 12 lactating pluriparous buffalo cows underwent repeated transvaginal follicular aspiration twice-weekly for seven sessions. OPU was carried out by using a portable ultrasound unit (Aloka SSD-500) with a 5 MHz sector scanner and a metal guide to fit 17 gauge needles both allocated in a properly designed vaginal guide. A vacuum pressure of – 40 mm Hg was constantly maintained and all visible antral follicles were punctured. The Cumulus Oocyte Complexes (COCs) were searched immediately after filtering the aspirated follicular fluid and aspiration medium and classified under 5 categories (A, B, C, D, E), according to a standard classification (Neglia *et al*., 2003). COCs were placed in 25 mM hepes buffered TCM 199 supplemented with 0.5 μg/ml FSH, 5 μg/ml LH, 1 μg/ml estradiol and 50 μM cysteamine (Gasparrini *et al*., 2000) in a portable incubator at 38.5°C and moved to the lab within 4 to 6 h. In the laboratory COCs were transferred into 50 μl droplets of a final maturation medium consisting of bicarbonate-buffered TCM 199 (B 199) with hormones and cysteamine in the same concentration previously described under medical oil. The droplets were incubated at 38.5°C for 22–24 h under a controlled gas atmosphere consisting of 5% CO_2 in humidified air. The day after IVM the oocytes were *in vitro* fertilized in 50 μl droplets of Fert-TALP, supplemented with 0.2 mM/ml penicillamine, 0.1 mM/ml hypotaurine and 0.01 mM/ml heparin. Sperm was treated using the swim-up procedure in a modified version of Ham's medium and resuspended in a final concentration of 2×10^6/ml. Fertilizing droplets were then incubated under the same gas atmosphere as for *in vitro* maturation. After 20–22 h presumptive zygotes were cultured in 20 μl droplets of SOFaaBSA in a modulation chamber with a gas atmosphere of 5% CO_2, 7% O_2 and 88% N_2. On day 5 (day 0 = IVF day) the cleavage rate was assessed and embryos were transferred into fresh droplets of the same medium for two further days of culture. Final embryo output (morulae + blastocysts) was evaluated on day 7 of culture. Some embryos were transferred fresh into opportunely synchronized buffalo heifers, by using a double injection of $PGF_{2\alpha}$ 12 days apart. Other embryos were vitrified using a method previously described (Neglia *et al*., 2001) and transferred after warming. Confirmation of pregnancy was obtained by rectal palpation of the genital apparatus at 45 and 120 days (after embryo transfer).

RESULTS

The cleavage rate (48.1%) as well as total morulae + blastocyst rate (19.6%) were lower than our standard results (Neglia *et al*., 2003), probably because a hemorrhagic

diarrhoea was diagnosed in the animals during the trial. A total number of 15 embryos were transferred: 7 blastocysts were transferred fresh and 8 after vitrification and warming. No pregnancies were assessed from fresh transferred embryos, whereas three pregnancies were found at 45 and 120 days after transfer of vitrified embryos (37.5%). Unfortunately one pregnancy was interrupted at 180 days, while the other two reached full term. In February 2003 the first two buffalo calves from vitrified embryos entirely produced *in vitro* were born.

DISCUSSION

As mentioned above, only three calves have been produced from frozen embryos (Galli *et al*., 1998), when zygotes were cultured into ligated ewe oviduct. In this way the sheep represents a temporary host, in which embryos can develop until day 7: this step improves the quality of the embryo, which grows under "normal" conditions and is more resistant to the cryopreservation process. In the buffalo species, the entire *in vitro* embryo production system has been developed by extrapolating information acquired in more studied species (cattle and sheep): for this reason the embryo nutritional requirements are unknown. Further studies are therefore necessary in order to optimize the *in vitro* development conditions of buffalo embryos. We underline the lack of pregnancies established by fresh embryos: this surprising finding may be explained by the higher sensitivity of buffalo embryos to environmental stress as compared with bovine (Neglia *et al*., 2003). However, the results obtained in this trial, demonstrate the possibility of using vitrified embryos produced *in vitro* in order to produce buffalo calves. This may in turn allow the application of OPU/IVEP technology to accelerate genetic progression through maternal lineage.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge F.lli Garofalo Farm for the support in the realization of this project.

REFERENCES

Boni, R., Roviello, S., Gasparrini, B. and Zicarelli, L., 1997. Pregnancies established after transferring embryos yielded by Ovum Pick-up and *in vitro* embryo production in Italian buffalo cows. *Proceedings of the V World Buffalo Congress*, Caserta, Italy 1997, 787–792

Galli, C., Duchi, R., Crotti, G. and Lazzari, G., 1998. Embryo production by Ovum pick-up in water buffalo. *Theriogenology*, **49**, 400

Galli, C., Crotti, G., Notari, C., Turini, P., Duchi, R. and Lazzari, G., 2001. Embryo production by Ovum Pick-up from live donors. *Theriogenology*, **55**, 1341–1357

Gasparrini, B., Neglia, G., Di Palo, R., Campanile, G. and Zicarelli, L., 2000. Effect of cysteamine during *in vitro* maturation on buffalo embryo development. *Theriogenology*, **54**, 1537–1542

Karaivanov, C., 1986. Comparative studies on the superovulatory effect of PMSG and FSH in the water buffalo (Bubalus bubalis). *Theriogenology*, **26**, 51–60

Looney, C.R., Lindsey, B.R., Gonseth, C.L. and Johnson, D.L., 1994. Commercial aspects of oocyte retrieval and *in vitro* fertilization (IVF) for embryo production in problem cows. *Theriogenology*, **41**, 67–72

Madan, M.L., Singla, S.K., Jailkhani, S. and Ambrose, J.D., 1991. *In vitro* fertilization in buffalo and birth of first ever IVF buffalo calf. *Proceedings III World Buffalo Congress*, Varna, Bulgaria 1991, 11–17

Madan, M.L., Singla, S.K., Chauhan, M.S. and Manik, R.S., 1994. *In vitro* production and transfer of embryos in buffaloes. *Theriogenology*, **41**, 139–143

Neglia, G., Gasparrini, B., Caracciolo di Brienza, V., Campanile, G., Spadetta, M. and Zicarelli, L., 2001. Cryopreservation of *in vitro* produced buffalo and bovine embryos by vitrification. *Proceedings VI World Buffalo Congress*, Maracaibo, Venezuela, 2001, 155–159

Neglia, G., Gasparrini, B., Caracciolo di Brienza, V., Di Palo, R., Campanile, G., Presicce, G.A. and Zicarelli, L., 2003. Bovine and buffalo *in vitro* embryo production using oocytes derived from abattoir ovaries or collected by transvaginal follicle aspiration. *Theriogenology*, **59**, 1123–1130

Totey, S.M., Singh, G., Taneja, M., Pawshe, C.H. and Talwar, G.P., 1992. *In vitro* maturation, fertilization and development of follicular oocytes from buffalo (*Bubalus bubalis*). *Journal of Reproduction and Fertility*, **95**, 597–607

Veterinary Research Communications, 28 (2004) 237–240

Effect of Total Replacement of Dietary Fish Meal by Plant Protein Sources on Early *post mortem* Changes in the Biochemical and Physical Parameters of Rainbow Trout

G. Parisi[1]*, M. de Francesco[1], F. Médale[2], F. Scappini[1], M. Mecatti[1], S.J. Kaushik[2] and B.M. Poli[1]
[1] *Dipartimento di Scienze Zootecniche – University of Florence, Italy;*
[2] *UMR NuAGe, INRA, 64310 St-Pée-sur-Nivelle, France*
* *Correspondence: Dipartimento di Scienze Zootecniche, Via delle Cascine n° 5, 50144 Firenze, Italy*
E-mail: giuliana.parisi@unifi.it

Keywords: fillet colour; plant protein; rainbow trout; *rigor* index

INTRODUCTION

The replacement of fish meal with plant protein sources is a major issue of interest in the aquaculture of Teleostei (Tacon, 1994). Although several studies have been undertaken over the last two decades, many questions remain about the feasibility of the total substitution of traditional protein sources. Moreover, the effects of the protein source on the quality characteristics of the product obtained have been little investigated (Médale, 2003).

The aim of this study was to evaluate the effect of a diet based only on plant protein sources on the behaviour of some biochemical and physical parameters during the first 24 h after death.

MATERIALS AND METHODS

Sterile triploid (to avoid possible effects of reproduction) rainbow trout (*Oncorhynchus mykiss*, Walbaum) at an average initial body weight of 19 g were divided into two groups, reared under the same conditions (water temperature 17 ± 1°C; dissolved oxygen 8.5 ppm) and fed two isoproteic (49% crude protein) and isoenergetic (21 kJ/g digestible energy) diets (two replicate for diet), different for the protein source. One diet contained fish meal (FM) as its protein source, while the second one contained only a mixture of plant protein ingredients (PP). The diets were adequately balanced for amino acidic content. After 157 days of rearing, the fish (having an average final body weight of 832 and 670 g, respectively for FM and PP) were killed by a blow to the head, after a cold narcosis in water and ice. 40 fish from each diet group were analysed during the first 24 h after death (10 fish at 0 h and 6 fish at each of the next

sampling times: 2, 3, 4, 5 and 24 h), while the remaining trout were retained for analysis performed each 24 h over the whole shelf life to determine other parameters (not presented here).

Immediately after death, all fish were weighed and total and standard length were measured. 30 trout from each group were covered in ice and stored at 1°C until the time of measurement. Immediately after death a sample of cranial epaxial muscle was withdrawn for 10 fish per diet and immediately frozen in liquid nitrogen for determination of ATP and its catabolites (Burns and Ke, 1985). The adenylate energy charge [AEC: (ATP + 0.5ADP)/(ATP + ADP + AMP)] was then computed from the concentration of ATP and its catabolites. The eye liquor and epaxial muscle pH and the dielectric properties (Fish Tester VI) were also measured for the same animal. The same parameters were also measured at each of the following sampling times. In addition, the *rigor* index (Bito *et al*., 1983) was measured. On the right fillet, withdrawn from the fish analysed immediately after death, the total length and colour in three different sites (cranial epaxial, cranial ipaxial, middle caudal) were measured using a Chromameter (Minolta CR-200; CIE L*, a*, b* system). Those measurements (fillet length and colour) were repeated on the same fillets stored at 1°C in a closed aluminium box at 2, 3, 4 and 24 h after death.

The data were analysed with a one way ANOVA (diet), while the data concerning the measurements obtained in the subsequent samplings on the same fillets (fillet length and colour) were analysed by analysis of variance using PROCMIXED by the SAS statistical package, considering diet, time of sampling and site of measurement (only for colour) as fixed factors and the subject as random factor.

RESULTS

The total replacement of fish meal by a mixture of plant protein sources over a long duration led to significant differences in trout growth. Preliminary statistical analyses showed that body weight did not have a strong influence on the behaviour of the physical and biochemical parameters considered. Evolution of the *rigor* index showed different behaviour between the two groups, with the trout fed the PP diet already showing a value of nearly 100% at 2 h after death, while the FM trout attained the whole rigor 3 h later (Table I). The observed behaviour is consistent with the AEC value (significantly lower in PP trout at 2 h after death) and with the evolution of fillet shrinkage. In fact, although the values obtained at 24 h for the fillet shrinkage were similar for the two groups, this parameter showed a more gradual behaviour in trout fed the FM diet. No differences were observed for the pH values, either in the eye liquor or in the epaxial muscle, or for the dielectric properties. As regards the colour, the site of measurement strongly ($p < 0.01$) affected all parameters. The lightness was modified during the 24 h, with higher values at the start and at the end of the evaluation period, but did not reveal any differences due to diet. The yellowness (b*)

TABLE I
Evolution of considered parameters in the two diets (FM and PP)

		0 h	2 h	3 h	4 h	5 h	24 h
Rigor index (%)	*FM*	—	51a	71a	79	100	100
	PP	—	96b	100b	92	100	100
AEC[1] (μmoles g^{-1})	*FM*	0.74	0.69b	0.65	0.41	—	—
	PP	0.74	0.57a	0.57	0.44	—	—
RFL[2] (%)	*FM*	100	93.81b	92.10B	90.11B	—	80.55
	PP	100	90.74a	87.50A	86.29A	—	79.01
Hue[3] (°)	*FM*	43.79A	45.54a	45.59A	44.96A	—	46.73A
	PP	48.45B	48.80b	49.42B	50.70B	—	50.68B

For a given parameter different letters in the same column indicate significant differences (A, B: $p < 0.01$; a, b: $p < 0.05$).
[1]Adenylate energy charge (AEC): (ATP + 0.5ADP)/(ATP + ADP + AMP).
[2]Relative fillet length (RFL), computed as percentage of the length measured at 0 h.
[3]Hue: $\tan^{-1}$ b*/a*. For the colour parameters the model of statistical analysis included the site of measurement.

was lower for FM than PP trout fillets, with more significant differences at 4 h after death (12.56 vs 14.56), justifying the differences recorded for the Hue values.

DISCUSSION

The protein source influenced the *rigor* index, AEC and fillet shrinkage behaviour, probably in relation to a difference in the efficiency of cell energy recovery in the hours immediately after death, induced by the diet composition. The differences observed in fillet colour seem to demonstrate the presence of an interaction between the protein source and the pigment used in the diet formulation.

The results obtained suggest a possible effect of the quality of the dietary protein source on the biochemical processes during early *post mortem* stages, with potential consequences on the shelf-life and quality characteristics of the final product.

ACKNOWLEDGEMENTS

Research financed by EU – Project Q5RS-2000-30068.

REFERENCES

Bito, M., Yamada, K., Mikumo, Y. and Amano, K., 1983. Studies on rigor mortis of fish. 1. Differences in the mode of rigor mortis among some varieties of fish by modified Cutting's method. *Bulletin Tokai Regional Fisheries Research Laboratory*, **109**, 89–96

Burns, B.G. and Ke, P.J., 1985. Liquid chromatographic determination of hypoxanthine content in fish tissue, *Journal of the Association of Official Analytical Chemists*, **68**(3), 444–448
Médale, F., 2003. Influence of feed and feeding practice on quality factors such as nutrient composition and eating quality of aquaculture species. In: *Proceedings of the TAFT 2003 Conference*, Reykjavik, 2003 (Published by The Icelandic Fisheries Laboratories), 375–378
Tacon, A.G.J., 1994. *FAO Fisheries Circular 881* (FAO, Rome)

Veterinary Research Communications, 28 (2004) 241–244

Effects of Dietary Conjugated Linoleic Acid (CLA) on Immunoglobulin Concentration in Sow Colostrum and Piglet Serum

R. Rossi, G. Pastorelli, V. Bontempo and C. Corino*
Department of Veterinary Sciences and Technology for Food Safety, Faculty of Veterinary Medicine, University of Milan, Via Celoria 10 – 20133 Milan, Italy
**Correspondence: Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Facoltà di Medicina Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy*
E-mail: carlo.corino@unimi.it

Keywords: piglets; immunoglobulin; colostrum; CLA

Abbreviations: CLA, conjugated linoleic acid; Ig, immunoglobulin

INTRODUCTION

Conjugated linoleic acids (CLA) is the collective name for a group of geometric and positional isomers of linoleic acid (C18:2) in which the double bonds are separated by a single carbon-carbon bond instead of a methylene group. CLA are known to have many favourable biological effects: anti-carcinogenic properties (Pariza *et al*., 1991), antioxidant properties (Ip *et al*., 1991), as well as a nutrient partitioning effect (Mersmann, 2001). They have been shown to protect against atherosclerosis in rabbits (Lee *et al*., 1994) due to the reduction of plasma HDL cholesterol and triglycerides (Corino *et al*., 2002a). Moreover, CLA isomers have also been shown to modulate the immune system (Sugano *et al*., 1998; Bassaganya-Riera *et al*., 2001; Corino *et al*., 2002b). The aim of the present study was to determine the effects of dietary CLA supplementation in sows during late gestation and lactation on colostrum immunoglobulins of classes G, A and M (IgG, IgA, IgM) and on the piglet serum immunoglobulin title.

MATERIALS AND METHODS

A total of 21 gestating sows were allotted according to weight and parity to one of three dietary treatments: 0% CLA (C) or 0.5% CLA from seven days before parturition until seven days postpartum (T1) and 0.5% CLA from seven days before parturition until weaning (T2). CLA powder contains 50% CLA isomers (CLA ONE powder, PharmaNutrients, Inc., 918 Sherwood Drive Lake Bluff, IL 60044, USA). During parturition, samples of colostrum were collected from each sow and were

immediately frozen at $-20^{\circ}C$ pending analysis of immunoglobulin concentrations. Blood samples were taken randomly from two piglets per sow at 21 d postpartum and 15 d post weaning for the determination of the immunoglobulin titre. The immunoglobulin concentrations (IgG, IgA, IgM) were determined based on the quantification of specific antigens according to the radial immunodiffusion method of Mancini *et al.* (1965). A commercial kit specific for swine (Bethyl Laboratories Inc., Montgomery, TX, USA) was used for the assay.

The results on the colostrum immunoglobulin concentration were analyzed by ANOVA using the SPSS (2001). Differences between means were tested using the Student–Newman–Keuls test. Data on serum parameters were analyzed by repeated measurements (ANOVA).

RESULTS AND DISCUSSION

The results on colostrum immunoglobulin concentration are shown in Table I. Colostrum IgG concentration was significantly higher ($p < 0.05$) for treated groups than control group (+30%). The concentration of IgA and IgM tended to be higher ($p < 0.10$) in the colostrum of sows given CLA. There were no differences between the T1 and T2 groups because sows were given CLA treatment at the same time, 7 d before farrowing. The serum IgG concentration in piglets at 21 and 35 d post-partum is reported in Figure 2. Serum IgG concentration at 21 d postpartum was significantly higher ($p < 0.05$) for treated groups (T1 and T2) than control group (C). At 35 d postpartum serum IgG was higher only for the T2 group as compared to control group. There were no significant differences in serum IgA and IgM in relation to the diet of sows or age of piglets, however the control group showed lower values than the treated groups.

The effects of dietary CLA supplementation on colostrum and serum immunoglobulin concentration confirmed the immunomodulatory effects of these isomers as previously reported by Corino *et al.* (2002) and Bontempo *et al.* (2002).

TABLE I
Colostrum immunoglobulin concentration in sows fed control (C) or CLA-supplemented diets from seven days before parturition until seven days postpartum (T1) and from seven days before parturition until weaning (T2)

Immunoglobulin group	C	T1	T2	SEM*	*p*
IgG, mg/dL	8241[a]	10896[b]	10348[b]	403.6	0.03
IgA, mg/dL	1208	1044	2096	184.7	0.06
IgM, mg/dL	628	792	915	98.1	0.08

*SEM standard error mean.
[a,b]Means without a common letter differ, $p < 0.05$.

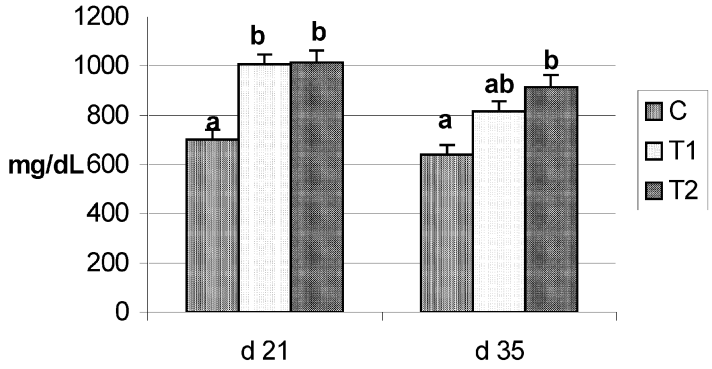


Figure 1. Serum IgG concentration at 21 and 35 days post-partum for piglets born from sows given control (C) or CLA-supplemented diets from seven days before parturition until seven days postpartum (T1) and from seven days before parturition until weaning (T2)

[a,b] Means without a common letter differ, $p < 0.05$

In particular we observed a positive effect at 21 d postpartum for piglets born from treated sows (T1 and T2) as compared to those born from non-treated sows (C). At 15 d post weaning there was a positive effect only for piglets born from sows fed CLA until weaning (T2). The T2 groups presented higher values for all the classes of immunoglobulins as compared to the control group in agreement with the results reported by Sugano *et al.* (1998).

Rooke *et al.* (2002) suggest that IgG synthesis by piglets is positively correlated to the amount of maternal IgG absorbed and thus reinforces the importance of an adequate IgG intake from colostrum. Also Krakowski *et al.* (2002) reported that high immunological values of colostrum determine the immunity of piglets not only in the suckling period, but also after weaning.

In conclusion, dietary CLA supplementation seems to favorably influence immune responses in piglets, suggesting an enhancement of host defenses against invading organisms.

This enhancement is likely to be particularly important in young animals that have not yet developed a specific immune response repertoire. Our findings suggest the dietary CLA should be further investigated as a possible alternative to the administration of growth-promoting antibiotics.

REFERENCES

Bassaganya-Riera, J., Hontecillas-Magarzo, R., Bregendahl, K., Wannemuehler, M.J. and Zimmerman, D.R., 2001. Effects of dietary conjugated linoleic acid in nursery pigs of dirty and clean environments on growth, empty body composition, and immune competence. *Journal of Animal Science*, **79**, 714–721

Bontempo, V., Magni, S., Pastorelli, G., Rossi, R., Sciannimanico, D. and Corino, C., 2002. Dietary

conjugated linoleic acid (CLA) influence immune response in suckling and weanling piglets. *Proceedings of the 53rd Annual Meeting of EAAP*, Cairo, 2002, **56**, N4.10

Corino, C., Mourot, J., Magni, S., Pastorelli, G. and Rosi, F., 2002a. Influence of dietary conjugated linoleic acid on growth, meat quality, lipogenesis, plasma leptin and physiological variables of lipid metabolism in rabbits, *Journal of Animal Science*, **80**, 1020–1028

Corino, C., Bontempo, V. and Sciannimanico, D., 2002b. Effects of dietary conjugated linoleic acid on some aspecific immune parameters and acute phase protein in weaned piglets. *Canadian Journal of Animal Science*, **82**, 115–117

Ip, C., Chin, S.F., Scimeca, J.A. and Pariza, M.W., 1991. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. *Cancer Research*, **51**, 6118–6124

Krakowski, L., Krzyzanowski, J., Wrona, Z., Kostro, K. and Siwicki, A.K., 2002. The influence of nonspecific immunostimulation of pregnant sows on the immunological value of colostrum. *Veterinary Immunology and Immunopathology*, **87**, 89–95

Lee, K.N., Kritchevsky, D. and Pariza, M.W., 1994. Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis*, **108**, 19–25

Mancini, G., Carbonara, A.O. and Heremans, J.F., 1965. Immunological quantitation of antigens by single radial immunodiffusion. *Immunochemistry*, **2**, 235–243

Mersmann, H.J., 2001. Mechanisms for conjugated linoleic acid-mediated reduction in fat deposition. *Journal of Animal Science*, **80**(2), 2130

Pariza, M.W., Ha, Y.L. and Benjamin, H., 1991. Formation and action of anticarcinogenic fatty acids. *Advances in Experimental Medicine and Biology*, **289**, 269–272

Rooke, J.A. and Bland, I.M., 2002. The acquisition of passive immunity in the new-born piglet. *Livestock Production Science*, **78**, 13–23

Sugano, M., Tsujita, A., Yamasaki, M., Noguchi, M. and Yamada, K., 1998. Conjugated linoleic acid modulates tissue levels of chemical mediators and immunoglobulins in rats. *Lipids*, **33**, 521–527

Veterinary Research Communications, 28 (2004) 245–247

Repeatability of Milk Clot Yield and Possibility of Reducing the Number of Controls Required to Identify the Individual Yield in the Buffalo

L. Zicarelli*, A. Potena, L. Amante and R. Di Palo
DISCIZIA, Faculty of Veterinary Medicine, "Federico II" University, Via F. Delpino, 1, Naples, Italy
**Correspondence: DISCIZIA, Sez. B. Ferrara, Facoltà di Medicina veterinaria, Via F. Delpino, 1, 80137, Napoli, Italy*
E-mail: zicarell@unina.it

Keywords: buffalo milk, cheese factory yield

INTRODUCTION

It has been demonstrated in previous reports (Potena *et al*., 2001a; Potena *et al*., 2001b; Zicarelli *et al*., 2001) that several subjects of "Bufala Mediterranea Italiana" showed a variable clot yield of between 22 and 30%, despite having the same milk chemical composition, the same diet and the same days open. Correlations between genetic variants and milk yield have not yet been observed for the buffalo species, but the possibility that one animal shows similar milk yield values in successive lactations, may be indicative of the utility of selecting for that characteristic. Currently, PKM supplies information on the quantity of mozzarella cheese produced/lactation, but this does not take into account the milk's cheese-making properties. Therefore, we thought it worthwhile to investigate the repeatability of the "milk yield" character and the possibility of classifying subjects into the categories of "high" or "low" milk yield. This may avoid monthly controls during lactation, because, in the case of a positive result, this character could be included in the selection programs for the species, whose milk is only used for cheese-making.

MATERIALS AND METHODS

During the first year of the trial, 1.5 litres of milk samples from the morning milking were collected monthly from 26 buffaloes. For 10 of these, other milk samples were also collected between 120 and 150 days open two times during the following year. The buffalo diet was kept constant throughout the trial (0.92 UFL/kg DM, 16% CP, 38% NDF, 31% NSC, 0.68% Ca and 0.38% P). Milk clot yield was evaluated by heating the milk at 42°C and adding 5 ml liquid clot (1:10000 titre) for each sample, according to the method described by Intrieri *et al*. (Intrieri *et al*., 1986). The clot was

weighed after 4 h, stored at 4°C, and weighed again 24 h from its constitution, upon whey elimination. Statistical analysis was performed by using ANOVA and analysis of the correlation coefficients (SPSS 11.0.1, 2002).

RESULTS

Significant values for the correlation coefficients were observed between the first and second year at 120 and 150 days for milk production (0.809; $p < 0.01$), milk clot yield at 4 (0.765; $p < 0.01$) and 24 (0.732; $p < 0.05$) hours after clot production and quantity of clot for each percent point of proteins (0.672; $p < 0.05$). If the value recorded in the first month is excluded (Table I) the grams of clot obtained from 1 litre of milk increased progressively during lactation ($r = 0.534$ and $r = 0.527$ respectively; $p < 0.01$). Between 121 and 180 days open the values were constant and not statistically different from those registered in other periods; the only variation was represented by a slight lowering registered between 241 and 270 days open. This variation was probably due to the coincidence with the period in which buffaloes were milked once daily and were near to dry themselves. The values in clot weight, both at 4 and 24 h, and at the different days open were always significantly correlated with the average of total lactation, excluding those at the end of lactation (>270 days). The highest correlation coefficients were obtained at 115 and 150 days. The mean of these values was highly correlated with the milk clot yield of total lactation ($r = 0.79$ and $r = 0.93$, respectively at 4 and 24 h; $p < 0.000$).

TABLE I
Average value of milk clot yield at different days open at 24 h

Days open	No.	Yield at 24 h
0–30	9	250.22^{cd}
31–60	30	218.37^{a}
61–90	24	223.52^{ab}
91–120	18	226.61^{abd}
121–150	22	235.79
151–180	23	239.74
181–210	17	247.52^{cd}
211–240	18	253.28^{c}
241–270	14	244.33^{b}
>270	11	266.06^{c}

Values with different superscripts are different ($p < 0.05$).

DISCUSSION

The grams of clot from 1 litre of milk were repeatable in the same buffalo during the two successive years, although the data need to be confirmed by further experiments. The value of the correlation coefficient between the average of the clot weight at 115 and 150 days open and the average of milk clot yield of each buffalo indicates that it is possible to reduce the number of controls for identification of the individual milk clot yield from nine to two.

REFERENCES

Intrieri, F., Barbieri, V., de Franciscis, G., Cavalieri, A. and Altiero, V., 1986. Proposta di un modello matematico per il pagamento del latte di bufala secondo qualità. *Atti XL Convegno Società Italiana Scienze Veterinarie*, Alghero, Sassari, Italia, 754–756

Potena, A., De Filippo, C., Bove, D., Cocca, T., Haubner, T. and Zicarelli, L., 2001. Resa alla caseificazione e parametri reologici del latte bufalino: risultati preliminari su latti individuali. *Proceedings I Congresso Nazionale sull'Allevamento del Bufalo*, Eboli, Italia, 236–240

Potena, A., Bove, D., Cocca, T., Haubner, T. and Zicarelli, L., 2001. Andamento di alcune componenti del latte di bufala in funzione della distanza dal parto: risultati preliminari. *Proceedings I Congresso Nazionale sull'Allevamento del Bufalo*, Eboli, Italia, 231–235

Zicarelli, L., Potena, A., De Filippo, C., Di Palo, R. and Campanile, G., 2001. Confronto tra bufale con differente resa alla caseificazione. *Proceedings I Congresso Nazionale sull'Allevamento del Bufalo*, Eboli, Italia, 241–246

Veterinary Research Communications, 28 (2004) 249–251

Detection of *Escherichia coli* Serotype 0157 in Beef Carcasses and Faecal Material

S. Albonetti*, M. Trevisani, S. Alonso Alvarez and R. Rosmini
Departement of Public Health Safety and Animal Pathology, School of Veterinary Medicine, University of Bologna, Italy
**Correspondence: Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, Sezione di Igiene e Tecnologia Alimentare, Facoltà di Medicina Veterinaria – Alma Mater Studiorum, Università di Bologna – Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy*
E-mail: salbonetti@vet.unibo.it

Keywords: beef, carcass, *Escherichia coli* serotype O157, faecal material, immunomagnetic separation

Abbreviations: API, identification system for Enterobacteriaceae and other Gram-negative rods; EHEC, enterohaemorrhagic *Escherichia coli*; HC, haemorrhagic coli; VTEC verocytotoxin producing *Escherichia coli*; ISO, International Standard Organisation; MFLP; MUG, 4-methylumbelliferyl-β-D-glucuronide; PBS, phosphate buffer saline; UV, ultraviolet lamp

INTRODUCTION

Enterohaemorrhagic (EHEC) *Escherichia coli* are recognised as human pathogens responsible for haemorrhagic colitis and in some cases, particularly in children under 6 years of age, cause of complications such as haemolytic uraemic syndrome and/or thrombotic thrombocytopenic purpura. The VTEC serotype O157 is most frequently reported in many countries and is frequently isolated from cattle and sheep, although other serotypes have been isolated from human cases. Human infection can also cause non-bloody diarrhea or result in no symptoms. Incidence of infection has presented a seasonal trend in many countries, with the summer months presenting higher prevalence of infection in both animals and humans. Many cases of infection have been reported in European and non-European countries and up to now 245 cases of VTEC infection have been reported to Enter-net Italia surveillance system. The aim of this paper is to evaluate the application of an official method for detecting typical (sorbitol-negative, glucuronidase negative) viable *Escherichia coli* O157 in food to microbial testing of beef carcasses and faecal samples at slaughterhouse.

MATERIALS AND METHODS

Ninety samples were collected in a slaughterhouse of Ravenna's province from December 2001 to January 2003. Samples were collected from carcasses and rectum content of forty-five slaughtered cattle. Tissue samples representing a total of 20 cm^2

were taken by cutting a slice of 5 cm^2 and maximum thickness of 5 mm off the carcass with a sterile blade from rump, flank, brisket and neck before chilling (2001/471/EC: Commission Decision of 8 June 2001). Tissue samples and faecal samples were put in sterile bags with 25 mL of Cary Blair Transport medium and kept refrigerated until analyses, which started within 24 h. The entire pool of tissue samples and 25 grams of faecal material were used to isolate *Escherichia coli* serotype O157 with the Official Laboratory Procedures MFLP-80 (March 2001) and MFLP-90 (April 1997) published on the Health Canada's website. In detail 25 g of faecal material or the tissue sample pool were homogenized in 225 mL of modified Triptic Soy Broth with Novobiocin and incubated at 42°C for 18–24 h (the first thirty samples) or 6–8 h (the latter sixty samples). An aliquot (1 mL) of the pre-enriched samples was used for the immunomagnetic separation with the Dynabeads® anti-*E. coli* O157 (Dynal Biotech, ASA, Oslo, Norway) following the procedure listed in the manufacturer's product insert. Immunobeads-bacteria complexes (50 μL) were spread over Modified Sorbitol MacConkey Agar with Tellurite, Cefixime and Cefsulodin (TCCSMAC) and Modified Hemorrhagic Coli Agar (mHC) with Sorbitol, MUG, tellurite and Cefsulodin. In detail, immuno-complexes were spread over one half of the plate with a sterile swab and were diluted further by streaking with a loop on the remaining two unstreaked quadrants. All isolated colonies with typical morphology were tested for ability to produce indole (ISO 7251:1993, 5.5 and 9.2.5–8; 9.2.6 modified about incubation temperature: 37°C instead of 45°C), fermenting cellobiose and O157 antigen using latex agglutination kit (Dry Spot *E. coli* O157, Oxoid, S.p.A., Garbagnate Milanese, Italia). Typical colonies (i.e. indole positive, cellobiose negative and positive to latex agglutination test) were further tested using the API 20E kit (BioMérieux Italia, Roma). The key for identification was that reported in Table 1 of Laboratory Procedure MFLP-80–2001.

RESULTS

The results obtained from the forty-five animals show that nineteen were positive for *Escherichia coli* O157. Eight of these were found positive only in faecal samples, four were found positive in both carcass and faecal samples and seven were found positive only in the carcass samples. Chance of detecting suspect colonies on differential media was affected negatively in the first 30 samples (four out of fifteen faecal samples and three out of fifteen carcass samples) by outgrowth of background micro-organisms, resulting confluent growth and/or diffusion of fluorogenic pigment. The problems was solved by reducing the incubation time in pre-enrichment broth to 6–8 h and improving the method of spreading the bacteria immuno-bead complexes.

DISCUSSION

Failure to recover sorbitol-negative and/or glucuronidase-negative colonies on differential agar plates may occur, because selectivity of differential agar plates is not

able to prevent outgrowth of confounding micro-organisms, which are common in beef carcasses and faeces. Major difficulties were found with the use of differential agar with MUG, because the water soluble pigment can diffuse rapidly in the agar. The choice of 6–8 h incubation time for pre-enrichment and careful spreading of immunobeads-bacteria complexes onto differential and selective agar, allowed very good recovery of isolated suspect colonies of *Escherichia coli* O157 from both beef carcasses and faeces. In conclusion the official laboratory procedures MFLP-80 (March 2001) and MFLP-90 (April 1997) adopted by Canada's Directorate of Health for Food can also be used for successful detection of typical *Escherichia coli* O157 from beef carcasses and faeces.

ACKNOWLEDGEMENTS

This study was supported by a grant from Bologna University, *ex* 60%, 2002.

REFERENCES

EC Commission Decision N. 471 of 08/06/2001, GUCE L 165, 21/06/2001
Enrichment of E. coli O157-Package Insert, http://www.dynalbiotech.com/
Dry Spot *E. coli* O157, Oxoid Limited, November 1998
Laboratory Procedure MFLP-80 (March 2001) Food Directorate's-Health Canada's, http://www.hc-sc.gc.ca/food-aliment

Veterinary Research Communications, 28 (2004) 253–255

Setting up a PCR Based Method to Trace Animal Species in Processed Meat Products

M.N. Losio[1]*, M.L. Ferrando[1], P. Daminelli[1] and F. Chegdani[2]
[1] *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna – Brescia.* [2] *Istituto di Zootecnica, Facoltà di Agraria, Università Cattolica del S. Cuore di Piacenza – Piacenza*
* *Correspondence: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, via Bianchi 7/9 25214 Brescia, Italy*
E-mail: nlosio@bs.izs.it

Keywords: meat traceability, protein analysis, PCR

INTRODUCTION

The identification of food species is becoming a very important issue for the assessment of food composition, necessary for the provision of proper consumer information. The increasing demand for transparency in the food industry derives either from socio-religious or economic reasons and has provoked a strong demand for appropriate detection methods that allow identification of the different components in processed food. The conventional methodologies used for the determination of species origin in food products are mainly based on immunochemical and electrophoretic analysis of proteins and on liquid chromatography techniques. However, immunological and electrophoretic methods cannot distinguish between closely related species and are often not suitable for food products with complex matrixes. Chromatographic techniques allow detection of differences in the percentages of fatty acids but are rather laborious. Recently, DNA-based techniques such as the polymerase chain reaction (PCR) or DNA hybridisation have been applied to identify meat species. These methods are sensitive and can still be used if the meat has been cured or autoclaved. Therefore, the aim of this study was either to optimise the extraction procedure of genomic DNA or to evaluate the suitability of a PCR method employing mitochondrial primers in detecting the authenticity of processed meat products.

MATERIAL AND METHODS

The tests were conducted with species-specific primers; in particular a region encoding tRNA e ATPase8 genes in the mitochondrial DNA was selected for bovine (forward: 5′-CACAATCCAGAACTGACAC-3′; reverse: 5′-GTAGGCTTGGGAA TAGTACGA-3′), the gene coding for subunit 1 of NADH dehydrogenase was selected for Gallus-Gallus (forward: 5′-CAAACATTGTGGGCCCTTTT-3′; reverse:

5′-AGTGGGAGGGGGACTCAAAT-3′) and two primer pairs were selected for swine; the first nuclear pair capable of amplifying the porcine endogenous retroviruses (FORWARD: 5′-CCCAGTACCCCTTCTAGGT-3′; reverse 5′-TCATCTA ATTGGAGGGTCAA) and the second amplifying a segment of the mitochondrial cytochrome b gene (forward: 5′-AAATCTCCCCTCAATGGTAT-3′; reverse: 5′-GGTAGTCCTATTATCGTGGG-3′).

The specificity of the selected primers was assessed using genomic DNA obtained from cell lines derived from specific and closely related species (turkey, sheep, goat). Moreover, the DNA extraction efficiency was evaluated (QIAamp DNA stool Mini Kit Qiagen S.p.a. -MI) related to different samples of processed meat products, starting from 200 mg of each sample. In particular the following products were selected: 15 samples of mortadella (5 known as Bologna-IGP), 20 samples of gelatine (10 known as having certified bovine origin) and 25 samples of sausages (10 of certified swine origin, 5 of certified poultry origin and 5 of certified bovine origin). The extraction was measured using a spectrophotometer (260/280 nm). Specific PCR reactions were performed on the extracted DNA. The results were analysed using standard agarose gel electrophoresis with ethidium bromide stain and then photographed in the UV region (254 nm). The results were compared with those obtained using the conventional ELISA test.

RESULTS

The results of the tests carried out to evaluate the selected primers and performed on selected cell lines have shown their suitability and the complete absence of cross-correlations. Moreover, the efficiency of selected extraction kit was assessed since all samples showed a value of 3 $\mu g \pm 1$ DNA/μl.

The results related to the authentication of food samples by PCR technique, compared to those obtained by ELISA test are reported in Table I. The results suggest the higher overall sensitivity of the PCR method for processed meat products. The gelatines, for example, were not suitable for the ELISA test as they were highly degraded. Furthermore, the PCR method appeared to be able to detect very low levels of other species different from those expected. Among the primers used, those specific to porcine endogenous retroviruses (PERV) appeared to be unsuitable for food analysis since they are only able to detect swine origin for unprocessed meat.

DISCUSSION

The results obtained showed the high suitability of PCR-based methods in detecting the authenticity of origin of processed meat products. This technique, which is able to reveal very low levels of species-contamination, appeared to be more suitable than the conventional ELISA test, which is highly affected by thermal treatments. This

TABLE I
Comparison between ELISA and PCR results

Food samples	PCR results[1]				ELISA results[1]		
	Bovine	Poultry	Swine*	Swine**	Bovine	Poultry	Swine
MORTADELLA							
5IGP	5/5	0/5	0/5	5/5	5/5	0/5	5/5
10	10/10	4/10	0/10	10/10	10/10	1/10	10/10
GELATINE							
10	10/10	0/10	0/10	0/10	ND§	ND§	ND§
10	7/10	0/10	0/10	3/10	ND§	ND§	ND§
SAUSAGES							
10	0/10	0/10	10/10	10/10	0/10	0/10	10/10
5	0/5	5/5	0/5	2/5	0/5	0/5	0/5
5	5/5	0/5	0/5	1/5	5/5	0/5	0/5
5	1/5	0/5	5/5	5/5	0/5	0/5	5/5

*Nuclear sequences; **mitochondrial sequences §not detected.
[1]Expressed as ratio between number of confirmed/number of examined samples.

diagnostic approach could become a useful tool in detecting possible frauds, offering more safety and security to the final consumers.

ACKNOWLEDGEMENTS

This work was supported by a grant from Italian Ministry of Health.

REFERENCES

Ashoor, S.H., Monte, W.C. and Stiles, P.G., 1988. Liquid chromatographic identification of meats. *Journal of the Association of Official Analytical Chemists*, **71**, 403–405

Jones, S.L. and Patterson R.L.S., 1985. Double antibody ELISA for detection of trace amounts of pig meat in raw meat mixtures. *Meat Science*, **15**, 1–13

Kim, H. and Shelf, L.A., 1986. Characterization and identification of raw beef, pork, chicken, and turkey meats by electrophoretic patterns of their sarcoplasms proteins. *Journal of Food Science*, **51**, 731–741

Veterinary Research Communications, 28 (2004) 257–259

Proposal of an Analytical Method for Determination of Residues of Organophosphorus Pesticides in Milk by GLC-NPD

G. Pagliuca*, T. Gazzotti, E. Zironi, N. Pavoncelli and R. Rosmini
Department of Veterinary Public Health and Animal Pathology – Alma Mater Studiorum – University of Bologna
**Correspondence: Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale – Sezione di Igiene e Tecnologia Alimentare – Alma Mater Studiorum – Università di Bologna, via Tolara di Sopra, 50 – 40064 Ozzano dell'Emilia, Bologna, Italy*
E-mail: pagliuca@vet.unibo.it

Keywords: milk, organophosphorus pesticides, GLC-NPD

INTRODUCTION

Organophosphorus pesticides are esters of phosphoric acid with different substituents; they are a heterogeneous group of compounds widely used within agriculture as insecticides for their broad spectrum of action.

Because of their short persistence in the environment, these compounds have replaced the organochlorine pesticides, however their toxicity, together with the possibility of bioaccumulation along the trophic chain, may represent a risk for human health (Tiecco, 2000). For these reasons we have developed an analytical method to identify and quantify the residues of organophosphorus pesticides in milk samples. There are already several methods available in the literature to determine the organophosphate contamination in vegetables, while very few have been developed regarding milk or other fatty matrices (Erney, 2000; Juhler, 1997).

Problems due to the complexity of these matrices, along with the presence of interferents, make the analyses of samples of animal origin difficult. For this reason extraction and purification are crucial, especially considering the difficulty in characterisation of organophosphorus pesticides having very different chemical structures and physical properties.

The aim of this work was to determine eight residues in milk: acephate, chlorpyriphos, chlorpyriphos-methyl, diazinon, methamidophos, methidation, phorate, pirimiphos-methyl. We have focused on these compounds because of their wide use in agriculture and their toxicity for human health.

MATERIALS AND METHODS

1 mL of acetone was added to 20 g of milk warmed to at least 20°C before the analysis. A mixture of 25 mL 1:4 acetone:acetonitrile was then added to the sample,

without shaking, and it was allowed to stand for 20 min. The sample was then shaken for 30 s, followed by centrifuging at about 4000 r.p.m. for 5 min.

The resulting liquid was decanted into a flask, and 2 mL of water was added to the milk solid followed by mixing with a spatula. Then 20 mL of acetone:acetonitrile mixture was added, and the sample was mixed again and extracted as before. The liquid phase was added to the previous one, and the extraction was repeated a third time. The liquid phases were shaken with 50 mL of dichloromethane in a separatory funnel and the phases were allowed to separate. The extraction was repeated two further times with 50 mL of dichloromethane.

After drying the extracted solution over anhydrous sodium sulphate for 30 min, the fraction was filtered using a paper filter and evaporated to dryness using a rotary evaporator. The sample was dissolved in 1 mL of acetonitrile and loaded onto a SPE C18 Monofunctional cartridge (500 mg/3 mL Phenomenex) previously conditioned with 5 mL of acetonitrile. The flask was then washed with another 2 mL of acetonitrile and 1 mL of 2-propanol.

The resulting 4 mL of sample was taken to dryness and redissolved in 200 μL of acetone. 2 μL of the obtained mixture was injected into the GLC system.

Gas chromatograph analyses were carried out using a HRGC Mega 2 8560 equipped with two NPD detectors (Nitrogen-Phosphorus Detector), combined with two capillary columns of different polarities: Zebron ZB-5 (30 m × 0.32 mm, i.d., 0.25 μm d.f.), and Zebron ZB-50 (30 m × 0.32 mm, i.d., 0.25 μm d.f.). The helium carrier gas was set at 1 cc/min.

The chromatographic temperature programme was first varied from 100°C to 130°C at 5°C/min, maintaining this temperature for 10 min, followed by a second increase of 5°C/min up to 220°C maintained for 7 min, then a third increase at 6°C/min to 274°C. The identification of pesticides was obtained through comparison of retention times from the two different columns. The method was validated and tested on samples of raw milk for human consumption. Before extraction, 1 mL of parathion-ethyl (concentration: 0.2 ppm) was added as an internal standard for quantitative determination. The method was developed in a UNI EN ISO 9001:2000 certified laboratory.

RESULTS AND DISCUSSION

This method proved to be effective for the simple and rapid quantitative determination and identification of eight organophosphorus pesticide residues in milk. The method was validated considering precision, accuracy and the limits of determination and quantification. Precision and accuracy were determined for milk spiked with three different concentrations of analytes. The values of relative standard deviation (RSD %) ranged between 0.10% and 0.38% while recoveries were more than 70%, except for methamidophos with a value of about 50%. This is probably due to the pesticide volatility which may result in a loss of residue during concentration (Erney,

2000). For each compound the limit of quantification (LOQ) is 5 ng/g (ppb) referred to milk, while the limit of detection (LOD) is 1 ng/g (ppb), estimated as the concentration of analyte which yields a signal-to-noise (S/N) ratio of at least 3/1.

These values permit quantification of the residues below the following legal limits: 10 ppb for chlorpyriphos-methyl, diazinon and methamidophos; 20 ppb for methidation and phorate; 50 ppb for pirimiphos-methyl (The European Commission, 2003). Parathion-ethyl was chosen as the internal standard because it should not be present in food since it was removed from the list of permitted bioactive substances in 2001 (Decreto Ministeriale 6 agosto 2001); moreover, this compound is stable and gives a good response to the NDP detector (Juhler, 1997). The method was tested on some raw milk samples. Among the samples analysed (more than 100) 30% were positive to acephate and chlorpyriphos contamination, but always below legal limits. Detection of positive samples confirms the importance of the control of OPPs contamination in milk, and illustrates the efficiency of this method.

ACKNOWLEDGEMENTS

The research was partially financed by "ex 40%" MIUR.

REFERENCES

D.M. 6 agosto 2001. *Gazzetta Ufficiale*. n. 272, 22 novembre 2001

Erney, R., 2000. Determination of organophosphorus pesticides in whole/chocolate/skim-milk and infant formula using SPE with capilary GC-NPD. *Food and Drug Administration Laboratory Information Bulletin ORA n.3929*, fda.gov/ora/scienceref/lib/lib3929.htm

Juhler, R.K., 1997. Optimized method for the determination of organophosphorus pesticides in meat and fatty matrices. *Journal of Chromatography A*, **786**, 145–153

The European Commission, 2003, Pesticide Residues: Maximum Residue Limits (MRLs), europa.eu.int/comm/food/fs/ph_ps/pest/index_en.htm

Tiecco, G., 2000. Ispezione degli alimenti di origine animale. *Edagricole*, 259–310

Veterinary Research Communications, 28 (2004) 261–263

Research on Bacteraemia in Cultured Rainbow Trout

A. Panebianco, A. Giuffrida*, F. Conte, G. Ziino, A. Minniti, and L. Iannuzzi
Department of Veterinary Public Health, Section of Inspection of Food of Animal Origin, Faculty of Veterinary Medicine, University of Messina, Messina, Italy
**Correspondence: Dipartimento di Sanità Pubblica Veterinaria – Sezione di Ispezione Alimenti di O. A. – Facoltà di Medicina Veterinaria, Polo Universitario dell'Annunziata, 98168 Messina, Italy*
E-mail: agiuffrida@unime.it

Keywords: trout, blood, muscle, seafood hygiene, bacteraemia

INTRODUCTION

As for several other species of interest in meat production, fish can suffer endogenous contamination of muscle as a consequence of a number of different environmental or catching stressors. This can be deduced from the studies of Herborg and Villadsen (1975) on the higher bacterial load and the lower quality and shelf-life of stressed trout, as well as from the research of Panebianco *et al.* (1993) on the isolation of *Clostridium perfringens* from the muscle of *Boops boops.*

In order to probe this issue we carried out research into the occurrence of bacteraemia in reared fish subjected to different catching methods. In previous work we showed the high incidence of bacterial isolation from muscle and blood of marine-cultured fish (*Dicentrarchus labrax*, *Sparus aurata*) and isolation of the same bacterial genus from muscle and blood in 10.81% of cases (Giuffrida *et al.*, 2003). In this work we report the results for cultured specimens of *Salmo gairdnerii.*

MATERIALS AND METHODS

39 specimens of *Salmo gairdnerii* were used for this study. Fish were caught with a small net and divided into three groups (A, B and C) on the basis of three different slaughtering methods: 11 fish (Group A) were slaughtered by resection of the medulla oblongata; 16 fish (Group B) were electrocuted (12 V per 15″) and 12 fish (Group C) were slaughtered by simple anoxia. Once the fish did not show any more vital behaviour, a blood sample was collected from the heart using a sterile syringe, after disinfection of the skin, and inoculated into three different blood-culture systems: Signal Blood Culture System (SBCS, OXOID); Hémoline Performance Anaérobie (BIOMÉRIEUX); Isolator™ 1.5 (OXOID); and into tubes of Salt Polimixin Broth (SPB, OXOID). SBCS blood-cultures were pre-incubated at 36°C for 1 h and then at 36°C for 24 h in a thermostatic agitator. The blood-cultures showing growth were

streaked onto plates of 3 different media: i) MacConkey agar (OXOID) plates which were incubated at 35°C for 24 h; ii) Pseudomonas agar base (OXOID plus Pseudomonas CFC Supplement) plates which were incubated at 30°C for 24 h; iii) Sulphite Polimixin Sulphadiazine agar (SPS, OXOID) plates which were incubated at 37°C for 48 h using anaerobic jars with a Gas Generating Kit (bioMerieux). The same media were used for the Isolator™ 1.5 blood-culture system, while the Hémoline Performance Anaérobie blood-cultures were only streaked onto SPS agar plates, incubated as indicated above. SPB enrichments were incubated at 30°C for 24 h and streaked onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (OXOID) plates, then incubated at 30°C for 24 h. A muscle sample was also aseptically drawn from each fish and divided into three sub-samples which were homogenised (1:9), respectively, with Peptone Buffered Water (PBW, OXOID), Reinforced Clostridium Medium (RCM, OXOID) and Salt Polimixin Broth (SPB, OXOID). PBW enrichments were streaked onto MacConkey agar (OXOID) plates which were incubated at 35°C for 24 h and onto Pseudomonas agar base (OXOID, plus Pseudomonas CFC Supplement) plates which were incubated at 30°C for 24 h. RCM enrichments were streaked onto SPS (OXOID) plates incubated at 37°C for 48 h using anaerobic jars with a Gas Generating Kit (BIOMERIEUX). SPB enrichments were streaked onto TCBS agar (OXOID) plates, incubated at 30°C for 24 h. After incubation all morphologically distinct colonies were picked, purified by restreaking onto tubes of Trypticase Soy Agar (TSA) (Oxoid), and identified using common bacteriological techniques and the Api 20E and API 20A systems (bioMerieux). Each sample was also subjected to necroscopy and a sterile swab was rubbed over each phlogistic focus and streaked directly onto plates of TCBS agar (OXOID) and Aeromonas agar (OXOID), which were incubated at 30°C for 24 h, as well as onto plates of Chromocult Coliform agar, which were incubated at 37°C for 24 h. The identification of morphologically distinct colonies was carried out as described above.

RESULTS

Isolations were carried out in 82.05% of blood samples and in 64.10% of muscle samples. The isolation from blood regarded the genera *Erwinia* (31.03%), *Enterobacter* (24.14%), *Shigella* (12.07%), *Serratia* (8.62%), *Flavibacterium* (6.90%), *Hafnia* (3.45%), *Acinetobacter* (5.17%), *Pseudomonas* (5.17%), *Sphingobacterium* (1.72%) and *Aeromonas* (1.72%). The isolations from muscle regarded the genera *Enterobacter* (48.00%), *Aeromonas* (28.00%); *Pseudomonas* (16.00%), *Chryseomonas* (16.00%), *Sphingobacterium* (16.00%), *Erwinia* (8.00%), *Hafnia* (8.00%), *Flavibacterium* (4.00%) and *Acinetobacter* (4.00%). In three cases (7.69%) the same bacterial genera were isolated from both blood and muscle; the genera were *Citrobacter freundii, Pseudomonas fluorescens/putida* and *Enterobacter cloacae.* Necroscopic examination showed three subjects with oral haemorrhagic foci from

which *Enterobacter agglomerans*, *Aeromonas hydrophila*, *Pseudomonas paucimobilis*, *Sphingobacterium multivorum* and *Pseudomonas cepacia* were isolated.

DISCUSSION

According to our results for marine cultured fish, the occurrence of bacteraemia in trout seems to be very frequent. The percentage isolation of the same bacterial genus from blood and muscle (7.69) is similar to that observed in *Dicentrarchus labrax* and *Sparus aurata* (10.81%) (Giuffrida *et al*., 2003). In these cases no correlation was observed with slaughtering methods, while other stressors (fish density, physical and chemical modifications of water, feed competition, etc.) could be influential in this regard. Furthermore, it is well known that these stressors can predispose fish to several pathologies sustained by opportunistic bacteria. The isolation of *Pseudomonas paucimobilis* and *Aeromonas hydrophila* from observed oral lesions, could demonstrate the occurrence of these stressors.

REFERENCES

Giuffrida, A., Conte, F., Ziino, G., Minniti, A., Iannuzzi, L. and Panebianco, A., 2003. Indagine sulle batteriemie in spigole e orate d'allevamento. *Atti Aassociazione Italiana VeterinariIgienisti*, **13**, 181–186

Herborg, L. and Villadsen, A., 1975. Cit. by Mayer, B.K. and Ward, D.R., 1991 in *Microbiology of Marine Food Products* (AVI Book, New York, 1991), 5

Panebianco, A., Minniti, A., Conte, F. and Iannuzzi, L., 1993. Ricerca di *Clostridium Perfringens* in Pesci Marini. *Archivio Veterinario Italiano*, **44**, 64–70

Veterinary Research Communications, 28 (2004) 265–267

Efficacy of Certain Disinfectants Towards Enteroviruses: Kinetics of *in vitro* and *in vivo* Inactivation

E. Pavoni[1]*, M.N. Losio[1], E. Bonetti[1], E. Cosciani Cunico[1] and C. Medici[2]
[1] *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna;*
[2] *Istituto di Microbiologia, Università degli Studi di Parma*
* *Correspondence: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Bianchi 7/9, 25124 Brescia, Italy*
E-mail: toxal@bs.izs.it

Keywords: enteroviruses, disinfectants, RT-nested PCR

INTRODUCTION

Foodborne infections are increasingly being recognized as being responsible for many human illnesses (Croci *et al*., 2001). Previously more attention was directed towards the detection of bacteria or parasites; however, the importance of viral infections is still underestimated by routine surveillance (Sair *et al*., 2001). Some types of food, such as shellfish, seem to play a major role in viral outbreaks, due to their high capacity of water filtration. Viruses are able to persist in mussels for a long period, even after their immersion in cleaned stalling water, and it has been demonstrated that they can survive decontamination processes used for bacterial agents (Richards *et al*., 1998). Italian law by decree no. 530 of 12/30/1999, regarding the sanitary rules for the trade of living mussels, does not provide for control of the absence of viral particles as sanitary inspection; it is only based on the quantitative determination of fecal bacteria. Nevertheless, it has been demonstrated that there is no correlation between bacterial and viral infections. Accordingly it is very important to establish new parameters capable of determining the tolerance of shellfish towards enteroviruses. For this reason, the D.C. no. 199/313/CE of 4/29/1999 indicates different procedures for the depuration of stalling water for molluscs which can eliminate enteric viruses. The purpose of this study was to evaluate the inactivation kinetics of enteroviruses with sodium hypochlorite, peracetic acid, chlorine dioxide, and hydrogen peroxide tested on viral dilutions and then inoculated in cell cultures. The efficacy of the inactivation process was evaluated by calculating the infectious titre. Inactivation kinetics were also studied using RT-nested PCR in contaminated live shellfish.

MATERIALS AND METHODS

Tests were performed using poliovirus type 1 (vaccine strain), coxsackievirus B5 and echovirus 30. Viruses were propagated in the BGM (Buffalo green monkey kidney)

cell line, which was amplified in MEM (Eagle's minimum essential medium Earle BSS) enriched with 10% foetal calf serum and 1% antibiotics (potassic penicillin G-1000 UI/ml, streptomycin sulphate-500 mg/ml and amphotericin B-2.5 mg/ml). The following disinfectant solutions were prepared: sodium hypochlorite ($0.2 \div 1$ mg/l), chlorine dioxide ($0.5 \div 2$ mg/l), hydrogen peroxide ($0.1 \div 0.5$ g/l), peracetic acid ($160 \div 640$ mg/l). 0.5 ml of each virus was added to 4.5 ml of disinfectants at the above concentrations. The solutions were then serially diluted by 10^{-1} and inoculated on a BGM monolayer which was incubated for five days. The infectious titre ($TCID_{50}$) was then determined using the Reed and Muench method (Reed and Muench, 1938). Experiments in the aquarium were conducted using poliovirus 1 in the presence of hydrogen peroxide which is known for its low toxicity towards shellfish. Forty molluscs were contaminated with the virus (final titre: $10^{4.50}$ $TCID_{50}$) for 24 h and were then transferred to a cleaned water pool containing H_2O_2 (0.375 g/l). Samples were then taken at pre-arranged time intervals and the residual titre was calculated by infecting the BGM cells and performing RT-nested PCR on shellfish tissue.

RESULTS

Poliovirus 1

The tests carried out using virus and sodium hypochlorite at different concentrations showed a clearly reduced infectious titre. In particular, concentrations of 0.6, 0.8 and 1 mg/l reduced respectively to 2, 3 and 4 Log. Inactivation kinetics of the virus with chlorine dioxide allowed detection of the fall in the titre from $10^{6.50}$ after 15 min to $10^{4.50}$ after 60 min at 1 mg/l. Peracetic acid reduced the infectious titre to $10^{5.24}$ after 10 min and $10^{4.24}$ after 60 min. Hydrogen peroxide had good inactivation efficacy under neutral pH conditions, while temperature did not influence its activity.

Coxsackievirus B5 and Echovirus 30

The use of sodium hypochlorite allowed detection of a decrease in virus viability 30 and 60 min after the first contact. The same behaviour was observed with chlorine dioxide and peracetic acid over a range of concentrations from 0.6 to 2.0 mg/l. Hydrogen peroxide (0.5 and 1 g/l) reduced the infectious titre by 3–4 logarithmic units after 6 h.

RT-nested PCR performed on water artificially contaminated with poliovirus type 1 confirmed the presence of virus in shellfish tissue 24 h after the addition of hydrogen peroxide and its complete absence after 48 h. In contrast, the inoculation of mussel homogenate on BGM after 24 h of disinfection did not allow detection of any cytopathic effect.

DISCUSSION

The results showed that the three types of viruses had similar kinetics when treated with sodium hypochlorite while, in the presence of chlorine dioxide, poliovirus 1 was more resistant than coxsackievirus B5 and echovirus 30. This difference was also confirmed on treatment with peracetic acid, while hydrogen peroxide, considering its low concentrations, was the most effective inactivator. The results showed that enteroviruses are resistant towards the most common disinfectants used in depuration systems. Following an initial decrease in the first titre count, viruses persist at a low concentration for a long period which is possibly affected by other factors such as pH and temperature. Positive samples found using RT-nested PCR could be due to the higher sensitivity of this method which can detect as few as 10 viral particles. Nevertheless, it has not yet been established whether this positivity can be attributed to viral infecting particles or to nucleic acid residues. Negativity revealed through PCR is very important for evaluation of depuration processes, but this could be affected by the large number of inhibitors that are potentially present within a product. For this reason it is necessary to test the inactivation kinetics of various viral serotypes and to prepare new experimental techniques that can more efficiently guarantee the complete depuration of these products.

REFERENCES

Croci, L., De Medici, D. and Fiore, A., 2001. Virus enterici veicolati da alimenti. In: G. De Felip. *Recenti sviluppi di Igiene e Microbiologia degli Alimenti*, 487–514

Reed, L.J. and Muench, H.A., 1938. Simple method of estimating 50% end points. *American Journal of Hygiene*, **27**, 493–497

Richards, G.P., 1998. Microbial purification of shellfish: a review of depuration and relaying. *Journal of Food Protection*, **51**, 218–251

Sair, A.I., D'souza, D.H., Moe, C.L. and Jaykus, L-A., 2001. Improved detection of human enteric viruses in foods by RT-PCR. *Journal of Virological Methods*, **100**, 57–69

Veterinary Research Communications, 28 (2004) 269–272

Microbiological Characteristics of Hamburgers and Raw Pork Sausages, and Antibiotic-resistance of Isolated Bacteria

D. Ranucci*, D. Miraglia, R. Branciari, V. D'Ovidio and M. Severini
Department of Food Science – Division of Safety and Quality of Food of Animal Origin, University of Perugia, Perugia, Italy
**Correspondence: Dipartimento di Scienze degli Alimenti, Sezione di Sicurezza e Qualità degli Alimenti di Origine Animale, Via San Costanzo, 06126 Perugia, Italy*
E-mail: ranucci@unipg.it

Keywords: hamburger, raw sausage, microbiology, antibiotic-resistance, food safety

INTRODUCTION

Meat preparations are exposed to microbiological risk due to their chemical-physical characteristics and the processing steps employed (Pizzin *et al*., 1998). Pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Salmonella spp.* are frequently found in these products (Pezzotti *et al*., 2001). The aim of this study is to assess the presence of these pathogens in meat preparations exclusively or mainly made with ground beef meat (hamburgers and meat patties) and raw pork sausages taken from the local market (Umbria region, Italy). The study also aims to evaluate the antibiotic-resistance of the eventually isolated *Staphylococcus spp., Staphylococcus aureus, Enterococcus spp.* and *Salmonella spp.* bacteria as well as to contribute to evaluation of the possible role of food of animal origin in the trasmission of antibiotic-resistant bacteria (WHO, 1997).

MATERIALS AND METHODS

Samples from 10 batches of beef hamburgers and 19 batches of meat patties (beef patties and beef/pork patties) were analysed. All of these products were produced at the meat department of various supermarkets. Samples from 25 batches of raw pork sausages were also analysed: four of these batches were produced in a semi-industrial plant and the remaining 21 in the meat department of different supermarkets. All beef and pork meat preparations sampled were taken directly from the display cases and transported in a refrigerated container to the laboratory within two hours. The samples were analysed using the following methods:

Pathogen isolation: a) *Staphylococcus spp.*: spread onto Baird-Parker + EGG (Oxoid) and incubated at 37°C for 48 h; b) *Staphylococcus aureus:* spread onto Baird-Parker + RPF (Oxoid) and incubated at 37°C for 48 h; c) *Escherichia coli:* spread

onto Coli ID (bioMèrieux) and incubated at 37°C for 48 h; d) *Salmonella spp.:* 25 g of each sample was pre-enriched in 225 ml of buffered peptone water and incubated for 18 h at 37°C. Pre-enrichment broth culture (0.1 ml) was then inoculated into 10 ml of Rappaport-Vassiliadis broth and incubated at 42°C for 24 h. Having completed the enrichment process, a loop (0.1 ml) of broth culture was then spread onto an XLD plate (Oxoid) and incubated at 37°C for 24 h. The suspect *Salmonella spp.* colonies were further tested (Kligler and urease) and serologically typed.

Antibiotic resistance evaluation: a) *Enterococcus spp.* vancomycin-resistant: 25 g of sample was put into 225 ml of VRE Broth Base + Meropenem (Oxoid) and incubated at 37°C for 24 h. A loop (0.1 ml) of broth culture was then inoculated into VRE Agar Base + Meropenem + Vancomycin (Oxoid) and incubated at 37°C for 24 h; b) 93 of the detected strains of *Staphylococcus spp.* and 18 strains of *Staphylococcus aureus* were randomly selected and tested for meticillin and vancomycin resistance; c) isolated strains of *Salmonella spp.* were tested against the following antibiotics by using the "disk diffusion method" according to Kirby-Bauer: Nalidixic acid (NAL), Ampicillin (AMP), Cefotaxime (CEF), Ciprofloxacin (CIP), Chloramphenicol (CLO), Gentamicin (GEN), Kanamycin (KAN), Streptomycin (STR), Sulfonamide (SUL), Tetracycline (TET), Trimethoprim (TRI), Neomycin (N), Colistin (CL), Enrofloxacin (ENR), Cefalotin (CF), Amoxycillin/Clavulanic acid (AMC). The resistance was interpreted according to the National Committee for Clinical Laboratory Standards tables (1997a, 1997b).

RESULTS

The results of the analyses performed for evaluation of the presence of pathogens are shown in Table I. *Staphylococcus aureus* was isolated in 21.1% of the hamburgers and meat patties (all samples containing less than 5×10^2 cfu/g). *Escherichia coli* was detected in 88.9% of the hamburgers and meat patties, with a concentration ranging from 5×10^2 to 5×10^3 cfu/g in 14.8% of the samples and higher than 5×10^3 cfu/g in 3.7% of the samples. *Salmonella spp.* was not detected in any of the above samples. *Staphylococcus aureus* was isolated in 47.1% of the sausages sampled (all samples containing less than 5×10^3 cfu/g and 11.77% containing more than 5×10^2 cfu/g). *Escherichia coli* were detected in 78.3% of sausages, with a concentration ranging from 5×10^2 to 5×10^3 cfu/g in 16% of samples and no samples had a concentration above 5×10^3 cfu/g. *Salmonella typhimurium* and *Salmonella rissen* were detected in two sausages samples, respectively. Regarding the antibiotic resistance, no *Enterococcus spp.* vancomycin resistant strains were detected and all the *Staphylococcus aureus* and *Staphylococcus spp.* strains tested proved non-resistant to vancomycin and meticillin. *Salmonella typhimurium* was resistant to (AMP), (STR), (SUL), and (TET), and *Salmonella rissen* to (STR), (SUL), (TET) and (TRI).

TABLE I
Distribution of *S. aureus* and *E. coli* and % of samples positive to *Salmonella spp.*

	Hamburger and meat patties (% of samples)			Raw pork sausages (% of samples)		
	<500 cfu/g	≥ 500–≤ 5000 cfu/g	>5000 cfu/g	<500 cfu/g	≥ 500–≤ 5000 cfu/g	>5000 cfu/g
S. aureus	100 (78.9 negative)	0	0	88.23 (52.9 negative)	11.77	0
E. coli	81.49 (11.1 negative)	14.81	3.70	84.00 (21.7 negative)	16.00	0
Salmonella		0			8.00	

DISCUSSION

The microbiological characteristics of the products considered in this study were similar to those reported by other authors (Del Gaudio *et al*., 2001, Pezzotti *et al*., 2001). The detection of *Salmonella spp*. in two of the examined pork sausages cannot be considered an unusual event, in agreement with other author (Cantoni *et al*., 1999; Mioni *et al*., 2000; Pezzotti *et al*., 2001) especially regarding *Salmonella typhimurium* (Bonardi *et al*., 2002), in that this is one of the serotypes most frequently detected in pig carcasses (Swanenburg *et al*., 2001). An interesting result of the study is the isolation of two *Salmonella spp*. strains that were resistant to four antibiotics. The risk that these micro-organisms can cause salmonellosis that cannot be treated with commonly used antibiotics is of great concern. Although the data are preliminary, it is reassuring to know that 100% of *Staphylococcus aureus* and *Staphylococcus spp*. proved non-resistant to vancomycin and meticillin and that no *Enterococcus spp*. vancomycin resistant were detected. The isolated strains of *Escherichia coli* were also tested for antibiotic resistance. However, due to the complexity of the results obtained, the data will be published at a later time. Finally, with regard to the consequence for the consumer, it must be stressed that the microbiological risk represented by meat preparations can only be dramatically reduced when these products are properly cooked.

REFERENCES

Bonardi, S., Brindani, F., Pizzin, G., Bacci, C., Cenci, A., D'Incau, M. and Liebana, E., 2002. Microbiological hazard of fresh pork sausages: result of a 1-year study. *Industrie Alimentari*, **41**, 782–788

Cantoni, C. and Soncini, G., 1999. Isolamento di salmonelle da alimenti di origine animale nel biennio 1997–1998. *Ingegneria Alimentare*, **3**, 25–29

Del Gaudio, F., Dessì, S., Sardu, C. and Sanna, A., 2001. Valutazioni microbiologiche di carni trite e preparazioni di carni ai sensi del D.P.R. 309/98. *L'Igiene Moderna*, **115**, 389–399

Mioni, R. and Pezzotti, G., 2000. Salmonella contamination of minced meat and meat products. *Atti X Congresso Nazionale Associazione Italiana Veterinari Igienisti*, 12–14 october 2000, Marsala., Italy, 207–211

National Committee for Clinical Laboratory Standards, 1997a. *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: tentative standard*, Waine, PA

National Committee for Clinical Laboratory Standards, 1997b. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard*, 4th Edition M7-A4, Villanova, PA

Pezzotti, G., Mioni, R., Grimaldi, M. and Ponzoni, A., 2001. Valutazione microbiologica di carni macinate e preparazioni di carne. *Ingegneria Alimentare*, **2**, 18–26

Pizzin, G., Bentley, S. and Maggi, E., 1998. Valutazioni microbiologiche di carni bovine e suine macinate. *Annali Facoltà Medicina Veterinaria Università di Parma*, **17**, 205–216

Swanenburg, M., Urlings, H.A.P., Snijders, J.M.A., Keuzenkamp, D.A. and Van Knapen, F., 2001. Salmonella in slaughter pig: prevalence, serotypes and critical control points during slaughter in two slaughterhouses. *International Journal of Food Microbiology*, **70**, 243–254

World Health Organization 1997. *The medical impact of antimicrobical use in food animals*, 13–17 October 1997, Berlin, Germany

Veterinary Research Communications, 28 (2004) 273–274

Rare Tumours in Domestic Animals: A Lipid Cell Variant of Urothelial Carcinoma of the Urinary Bladder in a Cow and a Case of Vesical Carcinosarcoma in a Dog

G. Borzacchiello[1], V. Ambrosio[1], L. Leonardi[2], R. Fruci[3], P. Galati[1] and F. Roperto[1]*

[1] *Department of Pathology and Animal Health, General Pathology and Anatomic Pathology Divisions – Faculty of Veterinary Medicine – Naples University "FEDERICO II" 80137 Naples Italy;* [2] *Department of Veterinary Biopathologic Sciences, Pathology and Hygiene Division, Faculty of Veterinary Medicine – Perugia University, Perugia Italy;* [3] *Practitioner.*

**Correspondence: Department of Pathology and Animal health, Via Delpino, 1, 80137 Naples, Italy*

E-mail: roperto@unina.it

Keywords: carcinosarcoma; lipid cell variant; urothelial carcinoma

INTRODUCTION

Neoplasms of the urinary bladder have a low incidence in domestic animals. In dogs they account for less than 1% of all reported neoplasms (Confer and Panciera, 2001). Cattle grazing on bracken fern-infested lands are at increased risk of suffering from tumours of the urinary bladder (Galati, 1997). It has been suggested that long term urine storage plays an important role in urothelial carcinogenesis since it could enhance the action of carcinogenic agents as it increases urine contact time with tissue (Caywood *et al*., 1980). It has been suggested that primary epithelial tumours of the urinary bladder occur more frequently in female than male dogs with an average age of 10 years (Crow, 1985).

About 76% of all urinary bladder tumours develop from the urothelium, and most of them are malignant (Caywood *et al*., 1980; Strafuss *et al*., 1975).

The recent WHO Histological classification of urinary bladder tumours in humans (Mostofi and Davis, 1999) describes some new variants of urothelial carcinoma that have not yet been reported in animals.

In an ongoing research program about the incidence of urinary bladder tumours at the Department of Veterinary Pathology at Naples Veterinary School, two new cases were observed: a lipid cell variant of urothelial carcinoma in a cow and a carcinosarcoma in a dog.

MATERIALS AND METHODS

Samples of the neoplastic urothelium were collected from bovine and canine urinary bladders. The samples were fixed in 10% buffered formalin and embedded in paraffin;

5 micron-thick sections were stained with haematoxylin-eosin (HE), periodic acid-Schiff (PAS) and Mayer mucicarmine stainings for neutral and acidic mucopolysaccharides. In addition, 8–10 μm thickened frozen sections were stained with saturated Sudan black B in 70% isopropyl alcohol and Oil Red O to detect intracytoplasmic lipids.

RESULTS

The lipid cell variant of urothelial carcinoma of the bovine urinary bladder was histologically characterized by clusters of urothelial cells exhibiting the appearance of signet-ring cells. Sudan Black B and Oil Red O stainings carried out on frozen sections showed intracytoplasmic lipids in many of these signet-ring cells. Signet-ring cells were also seen in the neoplastic proliferations.

The carcinosarcoma was characterized by two different components: multiple and diffuse nests of urothelial neoplastic cells were scattered over sarcomatous lesions.

DISCUSSION

The lipid cell variant of urothelial carcinoma in which clusters of lipocyte-like cells were scattered over the neoplastic urothelium suggests that they could be of epithelial origin. It is well known that the potentiality of the malignant urothelium is reflected in the wide range of variant morphologies of carcinomatous lesions.

The lipid cell variant, which is reported to be a rare variant of human urothelial carcinoma, has now been reported in veterinary medicine for a cow. Vesical carcinosarcoma is also very unusual for the dog.

REFERENCES

Caywood, D.D., Osborne, C.A. and Johnston, G.R., 1980. Neoplasms of the canine and feline urinary tracts. *Current Veterinary Therapy*, **7**, 1203–1212

Confer, A.W. and Panciera, R.J., 2001. The urinary system. In: M.D. McGavin, W.W. Carlton and J.F. Zachary (eds), *Thomson's Special Veterinary Pathology*, 3rd edn (Mosby, St. Louis, MO, USA), 235–277

Crow, S.E., 1985. Urinary tract neoplasms in dogs and cats. *The Compendium on Continuing Education for the Practicing Veterinarian*, **7**, 607–618

Galati, P., 1997. "Tumori dell'apparato urinario" in Diagnostica Istologica dei Tumori degli Animali – Fondazione Iniziative Zooprofilattiche e Zootecniche – Brescia, Italy, 233–253

Mostofi, F.K. and Davis, C.J., 1999. *Histological Typing of Urinary Bladder Tumours – WHO International Histological Classification of Tumours* (Springer, Berlin), 7–13

Strafuss, A.C. and Dean, M.J., 1975. Neoplasms of the canine urinary bladder. *The Journal of the American Veterinary Medical Association*, **166**(12), 1161–1163

Veterinary Research Communications, 28 (2004) 275–277

Aflatoxicosis and Vitamins A and E Supplementation in Sows: Immunological State of their Piglets

E. Cabassi*, R. Di Lecce, E. De Angelis, A. Fusari, A. Perillo and P. Borghetti
Department of Animal Health – General Pathology and Veterinary Anatomo-Pathology Section – Faculty of Veterinary Medicine, University of Parma, Via del Taglio, 8-43100 Parma, Italy
**Correspondence: Dipartimento di Salute Animale – Sezione di Patologia Generale e Anatomia Patologica Veterinaria, Università degli Studi di Parma, Via del Taglio, 8-43100 Parma, Italy*
E-mail: enrico.cabassi@unipr.it

Keywords: aflatoxins, peripheral lymphocytes CD4, CD8, CDγ/δ and CD21, piglets, vitamin A and vitamin E

Abbreviations: AF, aflatoxins; Vit A/E, vitamin A and vitamin E

INTRODUCTION

The authors' previous investigations showed that daily administration of 300 ppb of aflatoxin to sows from day 42–45 of gestation until weaning time, produced indirect intoxication of their piglets, leading to the exhibition of mild clinical signs of disease and histological patterns of altered cell-mediated immunity (Cabassi *et al*., 2002). Harvey *et al.* (1995) reported deficient levels of retinol and tocopherol in the tissue and serum of pigs affected by aflatoxicosis; they suggested that greater amounts of vitamins E and A are required by the body during the course of this disease and that both vitamins play a protective and detoxifying role in the presence of aflatoxins. Based on these considerations, the aim of our research was to assess wherether vitamin A and E supplements in AF contaminated feedstuff given to sows during gestation and lactation had a similar protective and detoxifying effect on their offspring. To verify this, the young animals' immune status was examined and signs of any adverse effects were sought by means of haematological and morphostructural examinations.

MATERIALS AND METHODS

Six polyhybrid [Duroc × (LW × L)], clinically healthy, pluriparous sows that had been artificially inseminated with semen from the same boar were used in the study. Two of them were kept as controls and the other four (AF + VitA/E) were kept under conditions of chronic aflatoxicosis by adding aflatoxins (B1,B2,G1,G2) to their daily

rations from day 42/45 of gestation until day 23 of lactation. All the animals were fed the same basic feedstuff that had been previously tested for mycotoxins. Aflatoxin intoxication was achieved by cultures of *Aspergillus flavus var. flavus* and *Aspergillus flavus var. parasiticus* seeded onto maize and rice grains. The intoxicated sows were supplemented with 60 UI of vitamin E and 11.000 UI of vitamin A per kg of feed.

Before euthanizing the animals, peripheral blood samples were collected from 4 piglets/sow on day 23 postpartum. The immunophenotypical evaluation of the main T/B lymphocyte subpopulations (CD4, CD8, CD21, CDγ/δ) was performed by flow-cytometry (De Angelis *et al*., 2001). Blood counts and serum biochemistry were also measured following traditional methods (Bonomi *et al*., 1997). Portions of the piglet's liver, kidneys, spleen, thymus and lymph-nodes were fixed in formalin, processed and stained following the routine procedure (Luppi *et al*., 2002) for post mortem examination. Samples of milk were taken from the sows on day 3 and 23 post-partum. The residual aflatoxins were measured by HPLC (Silvotti *et al*., 1997).

RESULTS

The piglets born from the two groups of sows considered (control and AF + VitA/E) were alive and viable, exhibiting clinical conditions throughout the trial. Post mortem examination revealed no patient alterations. Histological examinations showed structural changes of the liver, thymus, spleen and lymph-nodes of the AF + VitA/E sows. Signs of chronic disease were found in the liver with endocytoplasmic vacuolization and a greater amount of connective epimentation surrounding the lobules. Moderate lymphocyte depletion was observed in the splenic cortex and in the lymph-nodes. The malpighian splenic follicles showed signs of poor activations. Apart from slight individual differences, the blood counts were not particularly different between the two groups of piglets. Only MetHb was significantly lower in the AF + VitA/E group than in the controls. As shown in Table I, the CD4 and CD8 subpopulations were significantly reduced and so was the ratio between the intoxicated piglets and the controls. The residual AF measurements by HPLC showed traces of AF-M1 and 73 ng/l of

TABLE I
Trend of lymphocyte subpopulations (CD4, CD8, CD21, CDγ/δ – and the CD4/CD8 ratio) measured in piglets born from control sows and from intoxicated sows (AF + VitA/E). (Mean $\pm$ standard deviation)

	%CD4	%CD8	CD4/CD8	%CD21	%CDγ/δ
Piglets of control sows	32.9 ± 6.7	37.3 ± 8.1	0.85	36.6 ± 5.2	21.7 ± 3.8
Piglets of intoxicated sows AF + VitA/E	13.4 ± 2.1	25.9 ± 5.8	0.55	25.5 ± 7.1	5.12 ± 0.3

AF-M2 on day 3 of lactation and 77 ng/l of AF-M1 and 22 ng/l of AF-M2 on day 23 of lactation.

DISCUSSION

On the whole, the results of the study:

1) confirm the findings of our previous investigations that the administration of a pool of 300 ppb of AF to sows during pregnancy and lactation induces toxic effects on their piglets (Cabassi *et al*., 2002);
2) show that the factor causing intoxication of the piglets was the great amount of AF-M1 and AF-M2 they had received through their mothers' milk during their 23 days of life;
3) underscore the poor protective and detoxifying effects of vitamins E and A given to the sows as supplements in their daily rations contaminated by AF. The supplements did not prevent or restrict the morphostructural damage observed in the liver, thymus and lymph-nodes of the piglets that had been indirectly exposed to AF nor did they limit the abnormal cell-mediated immuno response. This may engender a greater susceptibility to disease in the animals.

ACKNOWLEDGEMENTS

The authors wish to thank Dott. M. Solfrizzo of the NRC of Bari and Dott. A. Biancardi of the I.Z.S.L.E.R. of Brescia for their availability and courtesy. This work was supported by grant from Italian MURST, COFIN 2000 (Prot. MM07578957_005)

REFERENCES

Bonomi, A., Quarantelli, A., Bonomi, B.M., Cabassi, E., Corradi, A. and Di Lecce, R., 1997. La contaminazione delle razioni per scrofe con aflatossine B1 e/o G1. Effetti sulle performances produttive della prole nelle fasi di accrescimento e di ingrasso. *La Rivista di Scienza dell'Alimentazione*, **26**, n.2, 42–60

Cabassi, E., Borghetti, P., De Angelis, E., Miduri, F., Losio, N. and Corradi, A., 2002. Aflatoxicosis in pregnant sows: cell-mediated response in their new-born piglets. *Proceedings of 20th Meeting of the European Society of Veterinary Pathology*, 234

De Angelis, E., Miduri, F., Borghetti, P., Martelli, P., Corradi, A. and Cabassi, E., 2001. Sottopopolazioni linfocitarie nel sangue periferico di suinetti da 1 a 10 settimane di età. *Atti della Società Italiana di Patologia ed Allevamento dei Suini,* **XXVII**, 209–214

Harvey, R.B., Kubena, L.F. and Elissalde, M.H., 1995. Effect of aflatoxins on tocopherol and retinol concentrations in growing barrows. *Agri Practice*, **16**, n.6, 12–14

Luppi, A., De Angelis, E., Losio, N., Lombardi, G., Miduri, F. and Cabassi, E., 2002. Immunotossicità da aflatossine in scrofe gravide. *Atti della Società Italiana di Patologia ed Allevamento dei Suini***,** XXVIII, 127–136

Silvotti, L., Petterino, C., Bonomi, A. and Cabassi, E., 1997. Immunotoxicological effects on piglets of feeding sows diets containing aflatoxins. *The Veterinary Record*, **141**, 469–472

Veterinary Research Communications, 28 (2004) 279–282

Improved Detection of *Coxiella burnetii* in Cows Milk by Immunomagnetic Separation and PCR

F. Capuano[1]*, A.G. Perugini[1], A. Parisi[2], C.O. Montagna[2] and M. Nilvelli[1]
[1] *Istituto Zooprofilattico Sperimentale del Mezzogiorno, sez. di Avellino;* [2] *Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, sez. di Bari*
* *Correspondence: Istituto Zooprofilattico Sperimentale del Mezzogiorno, Laboratorio di Biotecnologie, via Salute 2, 80055 Portici, Napoli, Italy*
E-mail: biotecnologie@izsmportici.it

Keywords: Coxiella burnetii, cows milk, immunomagnetic separation, PCR

Abbreviations: IMS, immunomagnetic separation; PCR, polymerase chain reaction

INTRODUCTION

The serum prevalence of *Coxiella burnetii* found in herds tested in the South of Italy (Landolfi *et al*., 2000; Capuano *et al*., 2001; Capuano *et al*., 2002) encouraged us to improve the performance of the PCR test to detect the *C. burnetii* pathogen in milk.

MATERIALS AND METHODS

Setting cow milk samples: one cow milk sample of each group was contaminated in activated cells infected with *Coxiella burnetii* (Dade Behring, Marburg GmbH, Germany). Each sample was serially diluted to ascertain the reliability of PCR, combined with other different techniques.

After contamination, group I specimens were packed by centrifugation at room temperature at 2000 G for 20 min. All the pellets were washed three times with PBS (phosphate-buffered saline, pH 7.4) and resuspended in PBS.

To detect DNA *C. burnetii* from group II specimens, the positive serum for Q fever (Dade Behring, Marburg GmbH, Germany) was diluted 1:1000 and added to artificially infected milk specimens. After one hour of incubation at room temperature, all samples were centrifuged at 10,000 G for 10 min, washed three times and resuspended in PBS. Immunomagnetic beads (IMB) covered with sheep antibody against rabbit IgG were added to the immunoprecipitates (Dynabeads M280) at the final concentration of 1.2×10^8 beads/ml.

Lastly, the specimens were determined by rotation for two hours at room temperature (Muramatsu *et al*., 1996).

The IMB belonging to group II samples were caught by trapping them against the surface of the Eppendorff like tube, using a magnetic particle concentrator (MCP-M,

DYNAL A.S.): then after removing the surplus fluid, all IMB were washed three times with PBS and lastly, resuspended in PBS.

Group III specimens were prepared for group II and then IMB was added and the samples were rotated for 18 h at 4°C to promote interaction between all the immuno-precipitates and the antibodies covering the IMB.

Before addition at the usual concentration to the group IV samples, all IMB were rotated for 18 h at 4°C after addition of the positive serum for *C. burnetii*.

After the addition of IMB, the group IV specimens were rotated for two hours at room temperature and they were then determined as for those of III group to collect all the IMB.

All the precipitates, resuspended in PBS, were boiled for 10 min and one portion of each sample was used for PCR assay.

PCR

PCR was performed according to the method designed for the amplification of *htp-AB* gene and one repeated region of the *C. burnetii* genome by Berri *et al.* (2000) using Trans-1 and Trans-2 primers (Fournier *et al.*, 1998).

RESULTS

We did not observe any non-specific amplification fragments in each group, although the direct IMB (group IV – Fig. 3) was the most reliable and rapid for detection of the pathogen *C. burnetii* as compared with classical and indirect (group II – Fig. 1) or modified IMS (group III – Fig. 2) methods.

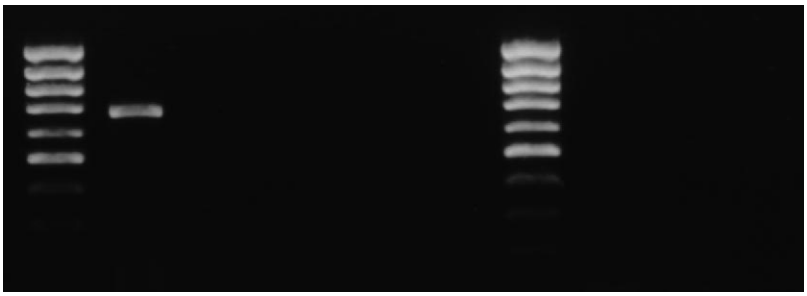


Figure 1. Group II (indirect IMS) – PCR. L: ladder 100 bp (Genenco, Life-Science, Milan). Coxiella serially diluted in milk: $1=10^{-2}$; $2=10^{-5}$; $3=10^{-8}$; $4=10^{-11}$; $5=10^{-14}$; $6=10^{-17}$, $7=10^{-20}$

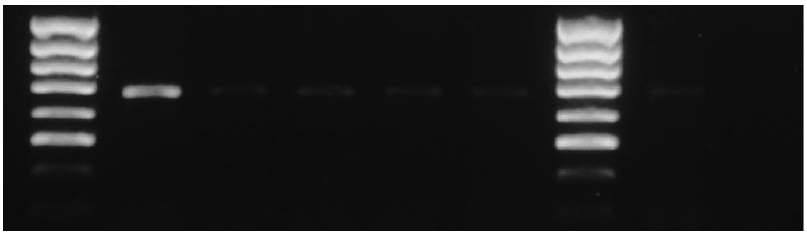


Figure 2. Group III (indirect and modified IMS) – PCR. L: ladder 100 bp (Genenco, Life-Science, Milan). Coxiella serially diluted in milk: $1=10^{-2}$; $2=10^{-5}$; $3=10^{-8}$; $4=10^{-11}$; $5=10^{-14}$; $6=10^{-17}$, $7=10^{-20}$

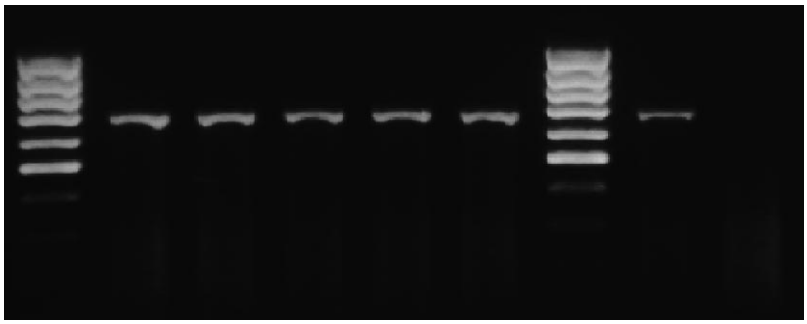


Figure 3. Group IV (direct IMS) – PCR. L: ladder 100 bp (Genenco, Life-Science, Milan). Coxiella serially diluted in milk: $1=10^{-2}$; $2=10^{-5}$; $3=10^{-8}$; $4=10^{-11}$; $5=10^{-14}$; $6=10^{-17}$, $7=10^{-20}$

DISCUSSION

The PCR test is very useful for the detection of *C. burnetii* in milk, but it is not always a diagnostically sensitive and reliable method, especially in milk collections, due to the presence of some substances that inhibit DNA amplification. The right choice of technique for sample preparation is fundamental for the improvement of detection PCR.

This study is so important to stress that both indirect and modified IMS and direct IMS are the only diagnostic methods able to detect *C. burnetii* in fully diluted samples (over 10^{-11} dilutions) and this may be because of the extended interaction time between IMB and the positive serum for Q fever in these techniques allows determination of the bacterial cells contaminating all the samples. Thus, direct IMS is really the most reliable method to detect *C. burnetii* by PCR, which is why only five hours are required to observe amplification.

REFERENCES

Berri, M., Laroucau, K. and Rodolakis, A. 2000. The detection of *Coxiella* from ovin genital swabs, milk and fecal samples by the use of single touchdowm polymerase chain reaction. *Veterinary Microbiology*, **72**, 285–293

Capuano, F., Landolfi, M.C. and Monetti, D.M., 2001. Influence of three types of farm management on the seroprevalence of Q fever as assessed by an indirect immunofluorescence assay. *Veterinary Record*, **149**(22), 669–71

Capuano, F., Landolfi, M.C., Rinaldi, L., Veneziano, V., Capelli, G. and Cringoli, G., 2002. Epidemiological survey of Q fever on dogs in an area of the Southern Italian Appennines and correlations with leishmaniosis and rickettsiosis. *Atti Società Italiana Scienze Veterinarie*, 197–198

Fournier, P.E., Marrie, T.J. and Raoult, D., 1998. Diagnosis of Q fever. *Journal of Clinical Microbiology*, **36**(7), 1823–1834

Landolfi, M.C., Esposito, A., Varlese, M.L., Fiore, E. and Capuano. F. 2000 *Atti Società Italiana Patologia Allevamento Ovini Caprini*, 127–130

Veterinary Research Communications, 28 (2004) 283–286

Effect of Temperature on *Betanodavirus* Infection in SSN-1 Cell Line

S. Ciulli[1]*, F. Ostanello[1], M. Battilani[1], S. Prosperi[1] and R.P. Hedrick[2]
[1] *Department of Veterinary Public Health and Animal Pathology, Alma Mater Studiorum Bologna University;* [2] *Department of Medicine and Epidemiology, University of California*
* *Correspondence: Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale – Alma Mater Studiorum Università di Bologna – Via Tolara di Sopra, 50, 40064 Ozzano Emilia, BO*
E-mail: sciulli@vet.unibo.it

Keywords: Betanodavirus, SSN-1, temperature, viral encephalopathy and retinopathy

Abbreviations: BFNNV, Barfin Flounder Nervous Necrosis Virus; FBS, Foetal Bovine Serum; L15 medium, Leibovitz medium; NNV, Nervous Necrosis Virus; RGNNV, Redspotted Grouper Nervous Necrosis Virus; SJNNV, Striped Jack Nervous Necrosis Virus; SSN-1, Striped Snakehead cell line; $TCID_{50}$, 50% tissue cultures infective dose; TPNNV, Tigger Puffer Nervous Necrosis Virus; VER, Viral Encephalopathy and Retinopathy; VNN, Viral Nervous Necrosis

INTRODUCTION

Viral Encephalopathy and Retinopathy (VER), otherwise known as Viral Nervous Necrosis (VNN), is responsible for frequent outbreaks with high mortality in a wide variety of marine fish species all over the world. The disease mainly affects the larval and juvenile stages which show neurological symptoms due to the typical vacuolating lesions in the brain, spinal cord and retina (Munday *et al*., 2002). The disease is caused by a virus of the *Betanodavirus* genus; seven viral species have been classified in this genus (van Regenmortel *et al*., 2000). This classification is based on the fish species from which the virus was isolated, but recently the isolation of the same virus from different host species has become very frequent (Ciulli *et al*., 2002; Castric *et al*., 2001). Cross-infection tests highlighted that the capacity of the virus to infect different species is mainly due to the environmental temperature at which the fish are farmed, thus at which the infection occurs, more than the host species itself (Totland *et al*., 1999). Using capsid protein gene analysis it was possible to classify four genotypes of *Betanodavirus*: Striped Jack Nervous Necrosis Virus (SJNNV), Tigger Puffer Nervous Necrosis Virus (TPNNV), Barfin Flounder Nervous Necrosis Virus (BFNNV) and Redspotted Grouper Nervous Necrosis Virus (RGNNV; Nishizawa *et al*., 1997). Iwamoto *et al*. (1999) demonstrated the different infection temperature ranges for the four genotypes. Both the infection and disease seem to be very temperature-dependent, in fact most of the outbreaks happen in the summer season.

This preliminary study elucidates the *in vitro* effects of temperature on

Betanodavirus infection in the SSN-1 cell line. NNV-infected SSN-1 cells incubated at 10–30°C after viral adsorption were observed for cytopathic effect and viral growth in the culture media supernatant and cell pellets were evaluated by titration.

MATERIALS AND METHODS

The nodavirus strain used in the experiments was isolated from symptomatic juvenile white seabass (*Atractoscion nobilis*) cultured in southern California.

SSN-1 cells were grown in 12.5 cm^2 flasks until the monolayers were confluent. The growth medium was removed and cells were inoculated with 250 µl of viral suspension ($10^{6,5}$ $TCID_{50}$/0.05 ml). After 1 h adsorption at room temperature the cells were washed twice with L-15 medium, then 3 ml of L15 medium containing 2% Foetal Bovine Serum (FBS) were added. Double flasks were incubated at 10°C, 15°C, 20°C, 25°C and 30°C and daily observed under a microscope to observe cytopathic effects. At 0, 12, 24, 48, 72, 96, 120, 168, 240 and 360 h post-infection both the free virus in the growth medium and the virus associated with the cells was titrated. Supernatants and cells were collected by scraping the bottom of the flasks. Cells were separated by centrifuging at 1800 *g* for 15 min and resuspended in 3 ml of L15 medium containing 2% FBS. Cells were homogenised in order to lyse them using syringes with a 25G needle. The titrations were performed in 96-well plates and $TCID_{50}$/0.05 ml were calculated according to Reed and Muench (1938).

RESULTS

A cytopathic effect was observed at 25°C two days post-infection and at 20°C three days post-infection. The observed cytopathic effect consisted of the typical cytoplasmatic vacuoles and it led to complete disintegration of the monolayer three days post-infection at 25°C and four days post-infection at 20°C. At 15°C we observed some alteration of the monolayer without the typical cytoplasmatic vacuoles. At 10°C we observed the death of all cells ten days post-infection. At 30°C, although in the first instance the monolayer was altered we didn't observe any relevant changes in the cells, even if they looked old and suffering from the high temperature.

The viral titre of cell samples incubated at 20°C and 25°C increased from $10^{4,376}$ $TCID_{50}$/0.05 ml to $10^{6,260}$ $TCID_{50}$/0.05 ml and to $10^{6,365}$ $TCID_{50}$/0.05, respectively and we observed a very similar increase in growth medium samples incubated at the same temperature. The viral titre remained constant for all days tested for samples incubated at 10°C. At 15°C we observed a small increase in the viral titre $>10^{5,3}$ $TCID_{50}$/0.05 ml only for cell samples seven days post-infection. At 30°C the viral titre decreased progressively during the test days, particularly in the supernatant where we could not detect the virus after 96 h (Fig. 1).

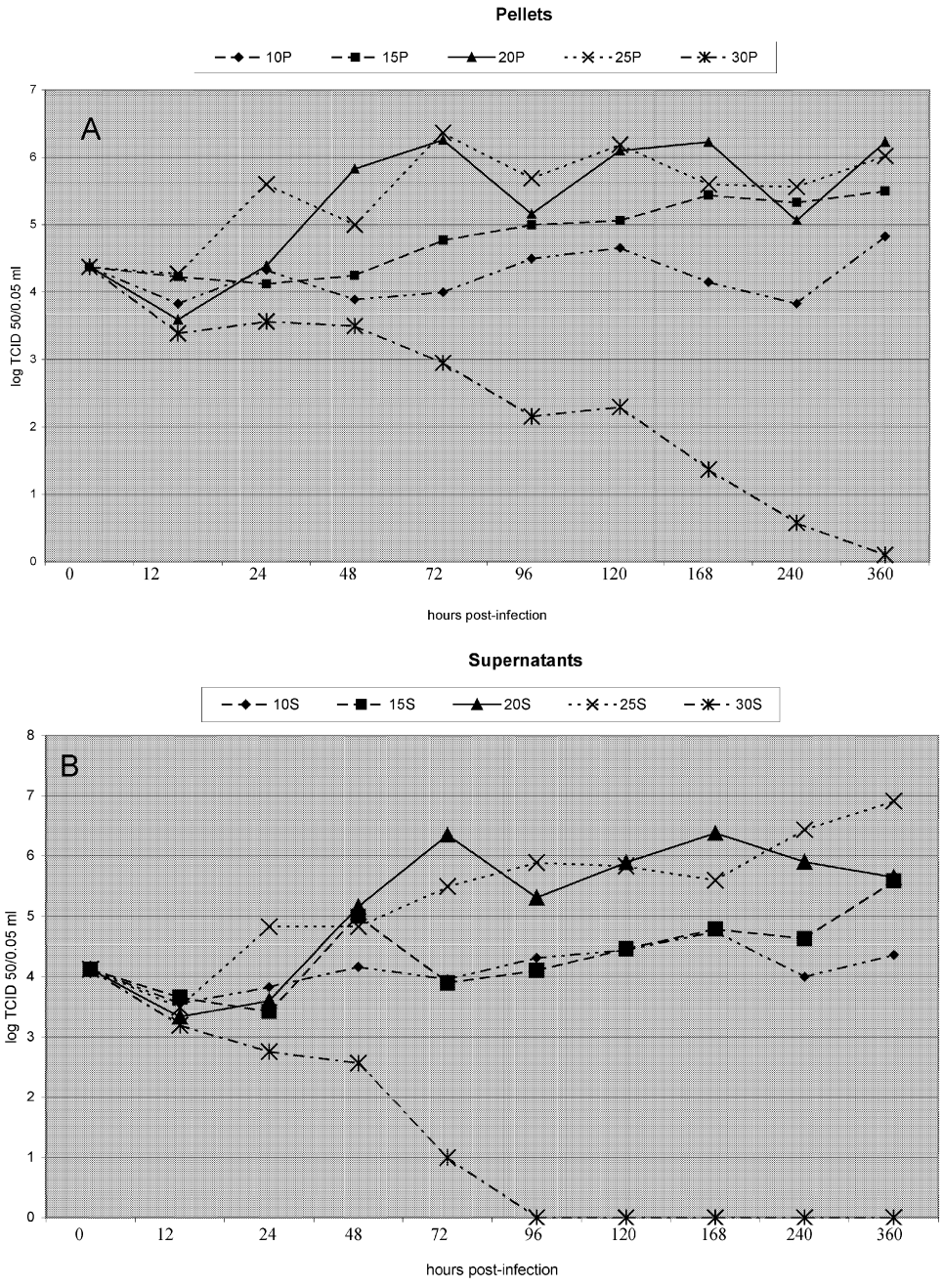


Figure 1. Viral titres in cell pellets (A) and in supernatants (B)

DISCUSSION

This preliminary study elucidates the *in vitro* effects of temperature on *Betanodavirus* infection in the SSN-1 cell line.

The viral titre remained constant for all days tested for samples incubated at 10°C, and this showed that no viral growth occurs at this temperature. 10°C was an unsuitable temperature for growth of the SSN-1 cell line, so cell death interfered with possible viral growth and we could not obtain any information about viral growth at this temperature. The high viral titre observed at the end of the experiment is probably due to the high resistance of the virus at this temperature.

15°C seemed to permit only partial virus replication in the cell, only a small amount of virus is produced and no typical vacuolisation can be observed in the cell monolayer. We suppose that even 15°C was an unsuitable temperature for growth of the SSN-1 cell line and this could interfere with viral growth.

The optimum temperature for growth is thus 25°C even if the virus also grows and gives a cytopathic effect at 20°C. 30°C does not seem to permit viral replication and causes a rapid viral inactivation. We also observed that the virus present inside the cell is more resistant to heat inactivation as compared to the virus free in the growth medium.

REFERENCES

Castric, J., Thiéry, R., Jeffroy, J., de Knkelin, P. and Raymond, J.C., 2001. Sea bream *Sparus aurata*, an asymptomatic contagious fish host for nodavirus. *Diseases of Aquatic Organisms*, **47**, 33–38

Ciulli, S., Florio, D., Scagliarini, A. and Prosperi, S., 2002. Analisi del gene della proteina del capside in *Nodavirus* proveniente da un cefalo (*Liza ramada*) di allevamento. Bollettino Società Italiana di Patologia Ittica, **34**, 14–22

Grotmol, S., Bergh, O. and Totland, G.K., 1999. Trasmission of viral encelopathy and retinopathy (VER) to yolk-sac larvae of the Atlantic halibut *Hippoglossus hippoglossus*: occurrence of nodavirus in various organs and a possible route of infection. *Diseases of Aquatic Organisms*, **36**, 95–106

Iwamoto, T., Mori, K., Arimoto, M. and Nakai, T., 1999. High permissivity of the fish cell line SSN-1 for piscine nodaviruses. *Diseases of Aquatic Organisms*, **39**, 37–47

Munday, B.L., Kwang, J. and Moody, N., 2002. *Betanodavirus* infections of teleost fish: a review. *Journal of Fish Diseases*, **25**, 127–142

Nishizawa, T., Furuhashi, M., Nagai, T., Nakai, T. and Muroga, K., 1997. Genomic classification of fish nodaviruses by phylogenetic analysis of the coat protein gene. *Applied Environment Microbiology*, **63**, 1633–1636

Reed, L.J. and Muench, H., 1938. A simple method of estimating fifty per cent end-points. *American Journal of Hygiene*, **27**, 493–497

Totland, G.K., Grotmol, S., Morita, Y., Nishioka, T. and Nakai, T., 1999. Pathogenicity of nodavirus strains from striped jack *Pseudocarnax dentex* and Atlantic halibut *Hippoglossus hippoglossus*, studied by waterborne challenge of yolk-sac larvae of both teleost species. *Diseases of Aquatic Organisms*, **38**, 169–175

Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.Z., Cartens, E.B., Estes, M.K., Lemon, S.M., Miniloff, J., Mayo, M.A., McGeoach, D.J., Pringle, C.R. and Wickner, R.B., 2000. Family Nodaviridae. In: *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*, 747–755. Academic Press, New York

Veterinary Research Communications, 28 (2004) 287–290

Evaluation of Endogenous Pig Retrovirus Expression and of Tumorigenicity in Nude Mice

M. Ferrari[1]*, A. Corradi[2], P.G. Petronini[3], A. Tosini[1], A. Toniolo[4], C. Robotti[1] and R. Moratti[5]

[1] *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia;* [2] *Dipartimento di Salute Animale, Università degli Studi, Parma;* [3] *Dipartimento di Medicina Sperimentale, Università degli Studi, Parma;* [4] *Dipartimento di Scienze Chimiche e Biologiche, Università degli Studi, Varese;* [5] *Servizi Veterinari, Azienda Sanitaria Locale; Brescia, Italy*

* *Correspondence: Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via A. Bianchi, 7/9, 25124 Brescia, Italy*

E-mail: substr@bs.izs.it

Keywords: PERV, antiretroviral drugs, expression inhibition, tumorigenicity *in vivo*

Abbreviations: DNA, desoxyribonucleic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction

INTRODUCTION

The current shortage of human allografts for transplantation may be overcome by using animal organs and tissue. Among the various donor species, pigs seem to be the best choice because they are available in a large number, can be housed in controlled specific pathogen free (SPF) environments and have anatomic and physiological features similar to humans. Despite these favourable conditions, xenotransplantation has two main problems. The first is the immunological incompatibility between pigs and humans which results in hyperacute rejection, and the second is the potential transmission of pathogens, especially endogenous retroviruses. The development of transgenic pigs with no or low expression of Gal $\alpha(1–3)$Gal antigen and with human cell surface complement regulators may overcome hyperacute rejection (Hammer *et al*., 1985). However, the potential transmission of pig endogenous retroviruses from the xenograft cannot be eliminated by modern gene knock-out technology and is still a potential risk. Therefore, the purpose of this study is to find some means of inhibiting PERV replication and production from two pig cell lines following treatment with some antiretroviral drugs commonly used in human medicine, as well as evaluating the potential tumorigenicity of these viruses for nude mice.

MATERIALS AND METHODS

Cells

The investigations were carried out on two cell lines derived from newborn pig kidneys (NSK; newborn swine kidney) and trachea (NPTr, newborn pig trachea),

respctively (Ferrari *et al*., 2003) both infected and able to produce A and B endogenous retrovirus classes. This infection was also transmitted, *in vitro*, to human cells.

Inhibition of retrovirus production

The following drugs were selected: nelfinavir (a protease inhibitor) (NFV, 0.030 μM), nevirapine (a non-nucleoside reverse transcriptase inhibitor) (NVP, 0.075 μM) and the four nucleoside analogs: stavudine (d4T, 0.042 μM), dideoxyinosine (ddI, 0.042 μM), lamivudine (3TC, 0.087 μM) and zidovudine (AZT, 5 μM).

The NSK and NPTr cell lines were cultivated for eight serial passages in culture medium containing a mixture of the above compounds. Viral production was evaluated in the supernatant medium of the last serial passage. The control used was culture medium from NSK and NPTr cells cultivated in absence of the drugs.

PCR and RT-PCR

Detection of the viral sequences in cell DNA and virus expression in the supernatant culture medium were evaluated using PCR and RT-PCR, respectively. The analyses were conducted employing primers specific for the conserved PERV *pol* region (Paradis *et al*., 1999)

Reverse transcription (RT) activity

This was carried out according to Barre-Snoussi *et al*., 1983. Briefly, the supernatants from NSK and NPTr cells were centrifuged at 200 *g* and the supernatants centrifuged again at 250,000 *g*. The viral pellets were resuspended in 5 μl of 2.5% Triton-X100 in order to disrupt viruses and were added to 50 μl of RT-cocktail solution (100 mM Tris HCl pH 7.9, 150 mM KCl, 1.2 mM $MnCl_2$, 4 mM DTT, 0.25 U/ml poly (rA) $p(dT)_{12-18}$, 60 μCi/ml ^3H TTP). After incubation at 37°C for 60′ a 120 mM NaPP in 60% trichloracetic acid (TCA) was added for 15′ at 4°C in order to induce precipitation. Samples were then transferred to a cellulose nitrate filter disc (0.45 μm) previously embedded with TCA (5%), rinsed, dried and re-suspended in scintillation fluid. The quantity of incorporated radioactivity was measured in a scintillation counter.

Tumorigenicity

NSK and NPTr filtered supernatants (0.45 μm) were centrifuged as above; the viral pellets were diluted in 400 μl of culture medium and inoculated by the subcutaneous

route to four nude mice (two for each supernatant type) at volumes of 200 μl for each animal (100 μl in each side). The mice were observed for 60 days, after which time they were euthanized. Tissue samples were collected from the inoculation areas at necropsy, as well as from organs and tissue of the abdominal, thoracic and neurocranic cavities. All samples were analysed using PCR for proviral integration of PERV. Furthermore, 5 μm paraffin inclusion sections were fixed in buffered formalin (pH 7.4) and then stained with the usual methods of histology and immunohistochemistry.

RESULTS

Inhibition of retrovirus production

Treatment with the selected anti-retroviral drugs did not completely inhibit virus replication. In particular, the RT analysis did not show a significant difference in virus production between the NSK cells exposed to anti-retroviral treatment and the control group (reduction in virus production of 3 fold). In contrast, virus replication appeared to be considerably lower (20 times) in the NPTr treated cells than in the un-exposed controls. Furthermore, the RT-PCR showed the persistence of viral particles in the supernatants of all treated and non-treated cells, and proviral sequences were also detected in the cell DNA by PCR.

Tumorigenicity

The inoculation of retroviruses in nude mice did not cause tumor growth and no malignant cells were observed in the organs or tissues of the injected animals on histological examination. Similarly, no viral sequences were detected in the tissues tested.

DISCUSSION

It has been shown that pig endogenous retroviruses can be transmitted to other cell-types (Soncini *et al*., 2001). However, their expression by human cells *in vitro* can be inhibited by treatment with drugs which are commonly used for retrovirus infection treatment in humans (Powell *et al*., 2000; Qari *et al*., 2001). In contrast, the results of this study showed that the expression of endogenous retroviruses from pig cells was reduced, but not completely inhibited, by antiretroviral treatment, suggesting some receptorial differences between the two species. On the other hand, the absence of tumorigenicity in the nude mice injected with retrovirus particles indicated that the

malignant features previously observed following inoculation either with NSK or NPTR cells were probably due to the cells themselves.

In conclusion, while the hyperacute rejection which may follow organ transplantation may be overcome by the use of transgenic pigs expressing human cell surface complement regulators, in contrast, retroviruses may incorporate the factors which regulate complement activation during their budding throughout the cell membrane with consequent prevention of virolysis induced by the natural antibodies in human serum.

As a consequence, the potential transmission of pig endogenous retroviruses during xenotransplantation may really represent a risk for public health.

ACKNOWLEDGEMENTS

This work was supported by a grant from Italian Ministry of Health.

REFERENCES

Barré-Snoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rozenbaum, W. and Montagnier, L., 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency sindrome (AIDS). *Science*, **220**, 868–871

Ferrari, M., Scalvini, A., Losio, M.N., Corradi, A., Soncini, M., Bignotti, E., Milanesi, E., Ajmone-Marsan, P., Barlati, S., Bellotti, D. and Tonelli, M., 2003. Establishment and characterization of two new pig cell lines for use in virological diagnostic laboratories. *Journal of Virological Methods*, **107**, 205–212

Hammer, R.E., Pursel, V.G., Rexroad C.E. Jr, Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D. and Brinster, R.L., 1985. Production of transgenic rabbits, sheep and pigs by microinjection. *Nature*, **315**, 680–683

Paradis, K., Langford, G., Long, Z., Heneine, W., Sandstrom, P., Switzerw., Chapman, L., Lockey, C., Onions, D., T.X.S. Group and Otto, E., 1999. Search for cross species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. *Science*, **285**, 1236–1241

Powell, S.K., Gates, M.E., Langford, G., Gu, M.L., Lockey, C., Long, Z. and Otto, E., 2000. Antiretroviral agents inhibit infection of human cells by porcine endogenous retroviruses. *Antimicrobial Agents and Chemotherapy*, **44**, 3432–3433

Qari, S.H., Magre, S., Garcia-Lerma, J.G., Hussain, A.I., Takeuchi, Y., Patience, C., Weiss, R.A. and Heneine, W., 2001. Susceptibility of the porcine endogenous retrovirus to reverse transcript and protease inhibitors. *Journal of Virology*, **75**, 1048–1053

Soncini, M., Scalvini, A.A., Bignotti, E., Pinoni, C. and Fouchier, R., 2001. Presenza di retrovirus endogeni in colture cellulari di origine suina e determinazione della eventuale trasmissione in sistemi cellulari di primati. *Atti LV Congresso Società Italiana Scienze Veterinarie*, 157–158

Veterinary Research Communications, 28 (2004) 291–293

Cloning and Expression of the Orf Virus F1L Gene: Possible Use as a Subunit Vaccine

L. Gallina*, A. Scagliarini, S. Ciulli and S. Prosperi
Department of Veterinary Public Health and Animal Pathology, University of Bologna,Italy
**Correspondence: Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale Alma Mater Studiorum Università di Bologna, Via Tolara di Sopra, 50-40064 Ozzano Emilia, BO, Italy*
E-mail: lauragallina@libero.it

Keywords: cloning, immunization, orf virus, subunit vaccine

INTRODUCTION

Contagious ecthyma is an infectious disease that commonly affects sheep, goats and occasionally humans. The disease is serious enough worldwide to be a cause of economic loss in sheep and goats, although this is poorly quantified (Haigh and Mercer, 1998). The causative agent of the disease is the orf virus, an epitheliotropic double-stranded DNA poxvirus of the genus *Parapoxvirus*. The importance of orf to immunologists and virologists lies in the fact that this virus can repeatedly infect sheep and goats in spite of the presence of a vigorous host immune and inflammatory response to infection. As a result of the immunomodulating activity of the virus no effective vaccine has been developed yet. Recent progress in analysis of the orf virus genome, the identification of its genes, the construction of deletion mutants as well as the generation of recombinants is leading to the selection of viral components that could mediate immuno-stimulating activity.

In this study we experimentally tried to assess the immunogenicity of the F1L protein in order to use it as a subunit vaccine.

MATERIALS AND METHODS

The full-length F1L gene of an Italian orf virus strain isolated from chamois (IT-TO) was amplified by PCR and cloned into the pCR T7/CT-TOPO vector fused with a six-histidine tag at the C terminus. The recombinant plasmid was propagated in *E. coli* TOP10F' grown in a selective medium. The expression was performed in 50 ml of *E. coli* BL21 transformed with 10 ng of the purified recombinant plasmid. Cells were grown overnight at 37°C with shaking and were then induced with 1 mM IPTG for 3 h at 37°C. The induced culture was centrifuged at maximum speed and the resulting pellet was analysed to detect expression of the recombinant fusion protein.

Western blot analysis was carried out using antibody against 6xHis epitope, polyclonal antibody from convalescent sera and monoclonal antibody 4D9 which binds to the viral neutralising epitope (Czerny *et al*., 1997).

The full length F1L protein was purified using a metal-chelate affinity chromatography under denaturing conditions. The eluates were analysed using pre-cast polyacrylamide gels and tested by Dot blot using monoclonal antibody 4D9.

Two rabbits were inoculated subcutaneously with 13.6 μg of the purified recombinant full-length F1L protein in Gerbu's adjuvant and were boosted 3 and 5 weeks after the first inoculation. The third inoculation was performed intravenously. The animals were bled at various intervals to check their antibody response against the subunit antigen by Western immunoblot analysis. Virus neutralization assays were performed by adding two-fold dilutions of preimmune and postimmune sera mixed with $10^3 TCID_{50}$ of wild type orf virus to TFO (ovine fetal testicular cells) cultured in Eagle's medium. The antibody titre was determined as the reciprocal of the serum dilution that neutralized more than 50% of CPE of the respective virus.

RESULTS

Western immunoblot analysis of induced culture pellets showed a band in the expected size range for the recombinant protein (42–46 kDa), moreover the recombinant peptide was detected using the 4D9 monoclonal antibody. Eluates obtained from protein purification and analysed by polyacrylamide gel electrophoresis showed a band in the expected size range for the recombinant protein which reacted against the monoclonal antibody 4D9 in a Dot blot.

Western blot assay using sera against the full-length recombinant F1L protein showed that the two rabbit seroconverted and the immunodominant protein of the IT-TO strain was recognized at a serum dilution of 1/100 and 1/1000 after the first inoculation and at 1/400 and 1/800 after the third immunization. Virus neutralization assays showed that both animals developed neutralizing antibodies against the viral strain IT-TO and the titers were 1:100 and 1:1000 respectively.

DISCUSSION

The results indicate that the method of choice for the production of the recombinant F1L protein is suitable for evaluation of the immunogenicity of the structural peptide. In fact, immunodetection of the recombinant peptide performed using pooled polyclonal sera from convalescent sheep and the 4D9 monoclonal antibody show that the neutralising epitope is conserved even after expression in BL21 cells.

These results demonstrate that rabbit immunization with the full-length F1L recombinant protein was able to stimulate the production of neutralizing antibody and suggest that the F1L protein is important for infection of the orf virus in cell

culture as previously shown for the homologue vaccinia virus H3L protein (Da Fonseca *et al*., 2000).

REFERENCES

Czerny, C.P., Waldmann, R. and Scheubeck, T., 1997. Identification of three distinct antigenic sites in parapoxvirus. *Archives of Virology*, **142**, 807–821

Da Fonseca, F.G., Wolffe, E.J., Weisberg, A. and Moss, B., 2000. Characterisation of the H3L envelope protein: topology and posttranslational membrane insertion via the C-terminal hydrophobic tail. *Journal of Virology*, **74**(16), 7508–7517

Haigh D.M. and Mercer, A.A., 1998. Orf. *Veterinary Research*, **29**, 311–326

Veterinary Research Communications, 28 (2004) 295–298

Duplex Polymerase Chain Reaction Assay to Screen for Feline Herpesvirus-1 and *Chlamydophila spp.* in Mucosal Swabs from Cats

F. Marsilio*, B. Di Martino, I. Aguzzi and I. Meridiani
Department of Scienze Biomediche Comparate, Faculty of Veterinary Medicine, University of Teramo, Italy
**Correspondence: Dipartimento di Scienze Biomediche Comparate, Facoltà di Medicina Veterinaria, Università di Teramo, Piazza Aldo Moro 45, 64100 Teramo, Italy*
E-mail: marsilio@unite.it

Keywords: cat, respiratory infections, diagnosis, duplex-PCR, RFLP

Abbreviations: FHV-1, feline herpesvirus type 1; FCV, feline calicivirus; PCR, polymerase chain reaction; U.R.T.D., upper respiratory tract disease; OMP, outer membrane protein

INTRODUCTION

Respiratory infections are widespread in the cat population and they are often associated with several pathogens such as FHV-1, FCV and *Chlamydophila spp.* (Gaskell and Dawson, 1998). The definitive diagnosis of feline herpesvirosis and feline chlamydophilosis requires the isolation of their aetiological agents, since serology alone does not allow us to distinguish infected animals from vaccinated ones. FHV-1 isolation using cell cultures is easily performed, even if some cats can show negative results because of (i) inadequate amount of virus present in the ocular and/or pharyngeal swabs, (ii) presence of antibodies in the extra-cellular liquids able to inhibit viral replication *in vitro*, (iii) contemporaneous presence in the swabs of FCV that could disguise the cytopathic effect induced by FHV-1. As regards *Chlamydophila felis* these problems are increased by the objective difficulty of the isolation that can be performed through inoculation of embryonate eggs of chicken. To remove these disadvantages it is useful to utilize the techniques of molecular biology, such as the polymerase chain reaction, which is able to detect specific sequences of DNA. Furthermore, it is possible to proceed from the amplicons to the identification of the chlamydofiles after treatment with restriction enzymes. The aims of the current research are (i) to report the results of a study performed by duplex-PCR specific for FHV-1 and *Chlamydophila spp.* in a population of 40 cats with U.R.T.D.-related symptoms in order to appraise the prevalence of two of the most common agents associated with respiratory infections, and (ii) to identify *Chlamydophila felis* using restriction enzymes.

MATERIALS AND METHODS

Mucosal swabs

From October 2002 to January 2003 forty mucosal samples, each composed of a pharyngeal and conjunctival swab, were collected from cats with respiratory symptomatology related to U.R.T.D. The samples were collected from private and public Italian veterinary clinics in the Isernia, Ascoli Piceno and Teramo areas. At the time of collections an anamnestic card was filled in with information about the type of swabs collected, the clinical signs and the possible pharmacological or vaccinal treatments for each cat examined. It was not possible to collect pharyngeal swabs from three of the cats. The remaining 77 samples were collected using suitable sterile swabs which were dipped immediately after collection into DMEM medium with added antibiotics. After this the samples were kept at $+4^{\circ}$C during transport to the laboratory and were then stored at -80°C until testing.

Nucleic acid extraction

Nucleic acid extractions were performed using a commercial kit (QIAmp UltraSens Virus, Qiagen), useful for the extraction of DNA and RNA from liquids containing few cells. The method followed was the one recommended by the manufacturer. The treated samples were first submitted to single PCR specific for FHV-1 and *Chlamydophila spp.* and then to the duplex-PCR.

Single PCR specific for FHV-1

The FHV-1 target sequence was 321 bp in length and included the TK gene coding for thymidine-kinase enzyme. The following pair of primers was applied: *FHV-F: 5′-TGT CCG CAT TTA CAT AGA TGG-3′ sense primer; FHV-R: 5′-GGG GTG TTC CTC ACA TAC AA-3′ antisense primer.*

Single PCR specific for Chlamydophila spp.

For *Chlamydophila spp.* the target sequence was 590 bp in length and it included the OMP2 gene encoding for the outer membrane protein (Hartley *et al.*, 2001). The following pair of primers was applied: Chla AF: 5′-ATG TCC AAA CTC ATC AGA CGA G-3′ sense primer; Chla AR: 5′-CCT TCT TTA AGA GGT TTT ACC CA-3′ antisense primer.

Duplex-PCR for Chlamydophila spp. *and FHV-1*

Simultaneous amplification reactions were performed in 50 µl of total volume containing 4 µl of viral and bacterial DNA, HotMaster Taq 1X buffer, 200 mM each of dATP, dTTP, dGTP and dCTP, 2.5 U HotMaster Taq DNA polymerase (Eppendorf), 0.04 mM FHV-F and FHV-R, 0.2 mM Chla-AF and Chla-AR. The reaction mixture was subjected to 40 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C followed by final extension of 7 min at 72°C. PCR products were subjected to electrophoresis through a 2% agarose gel. After staining with ethidium bromide the visible bands of expected size were visualized with ultraviolet transillumination.

PCR-Restriction fragment length polymorphism analysis

PCR products resulting from amplification of the OMP2 gene of *Chlamydophila spp.* were subjected to restriction endonuclease analysis using the HINDIII enzyme (Biolabs, New England) to proceed to species identification. HINDIII enzyme recognizes the AiAGCTT sequence and is able to cut the PCR product positive for *Chlamydophila felis* into two fragments of 122 bp and 468 bp in length, thus discriminating *Chlamydophila felis* from other species. Digested fragments were analyzed using 3% agarose gel electrophoresis and visualization with ethidium bromide staining and ultraviolet transillumination.

RESULTS

Twenty-four conjunctival swabs (60%) and twenty-one pharyngeal swabs (56.70%) were positive for FHV-1 and only nine conjunctival swabs (22.50%) were positive for *Chlamydophila spp.* The swabs positive using single PCR were also positive using duplex-PCR and all swabs positive for *Chlamydophila spp.* were positive for FHV-1. Furthermore, eight animals were positive for both pathogens. Only fourteen cats had been vaccinated against FHV-1 and none against *Chlamydophila felis.* The results divided according to the vaccination against FHV-1 reveal that most of the positive swabs originated from animals that had not been submitted to vaccination. As regards *Chlamydophila spp.* the highest number of positive swabs originated from animals treated with non-specific antibiotic therapy (7/27). Another interesting aspect concerns the type of swabs collected. In fact only the ocular swabs were positive for *Chlamydophila spp.*, while the results were more complicated for FHV-1. Indeed, three animals had positive pharyngeal swabs, four had positive ocular ones and eighteen had positive results for both. Finally, the restriction analysis performed using HIND III enzyme on the amplicons positive for *Chlamydophila spp.* showed the same pattern and therefore was concluded to belong to the *Chlamydophila felis* species.

DISCUSSION

The data coming from this investigation underline the fact that FHV-1 and *Chlamydophila spp.* are present in mucosal swabs collected from cats with respiratory symptomatology living in the different geographical areas subject to investigation. Another interesting aspect is that related to the type of sample sent to the laboratory. In fact, it must be underlined that while for *Chlamydophila spp.* positive results were found only in swabs from conjunctiva, for FHV-1 the infection was diagnosed on swabs collected from both conjunctiva and/or tonsils. Restriction analysis applied to positive amplified sequences highlighted a substantial uniformity in the nine strains, and therefore, all are identifiable as belonging to the *Chlamydophila felis* species. The correspondence of the results obtained using single specific PCRs towards each pathogen and those from duplex-PCR shows that this last method has identical diagnostic features to the specific PCR. The use of a single test which is able to identify both pathogens simultaneously, is particularly important, since it permits early diagnosis, using a test that has a higher sensitivity and specificity than the traditional techniques.

REFERENCES

Gaskell, R. and Dawson, S., 1998. Feline Respiratory disease. In: C.E. Greene (ed), *Infectious Diseases of the dog and cat*, W.B. Saunders Company, Philadelphia

Hartley, J.C., Kaye, S., Stevenson, S., Bennett, J. and Ridgway, G., 2001. PCR detection and molecular identification of Chlamydiaceae species, *Journal of Clinical Microbiology*, **39**, 3072–3079

Veterinary Research Communications, 28 (2004) 299–301

Evaluation of Angiogenesis by Morphometric Analysis of Blood Vessels in Dysplastic and Neoplastic Lesions of Canine Gingiva

M. Martano*, P. Maiolino, M. Cataldi and B. Restucci
*Department of Pathology and Animal Health, Section of Pathologic Anatomy, Faculty of Veterinary Medicine – *Faculty of Biotechnology Science, University Federico II Naples – Italy*
**Correspondence : Dipartimento di Patologia e Sanità Animale, Settore di Anatomia Patologica, Facoltà di Medicina Veterinaria, Università degli Studi di Napoli Federico II, Via Delpino, 1–80137 – Napoli – Italy*
E-mail: manuela.martano@unina.it

Keywords: angiogenesis, dysplasia, tumours, gingiva, dog

INTRODUCTION

Angiogenesis, the formation of new vessels from the pre-existing vascular bed, is a key event in physiological processes, such as embryogenesis and cyclic changes of the ovary and endometrium (Dvorak, 1995), as in pathologic processes, in particular in oncology. Neoplastic cells, having a high proliferative rhythm and increased metabolic requirements, are able to induce the formation of new vessels by angiogenesis (Folkman, 1990). Therefore, a correlation between neoplastic proliferation and angiogenesis has been demonstrated in several tumoral types (Vartanian *et al*., 1995). In addition, the formation of new vessels is also correlated with the metastatic potential of a neoplasm.

For these reasons, the microvessel density (number of microvessels per mm^2 of neoplastic tissue) which is the direct expression of the angiogenic potential of a neoplasm, together with the evaluation of vascular parameters (area and perimeter, in particular) are considered powerful prognostic tools in both human and in veterinary medicine (Fox, 1996; Weidner, 1998; Maiolino *et al*., 2000, 2001; Restucci *et al*., 2000, 2002).

MATERIAL AND METHODS

30 samples of gingivas, six normal and 24 pathologic, were examined; these were classified, using the Pindborg criteria (1997), as medium dysplasia (7), severe dysplasia (9) and carcinomas (8). All samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. 4 μm thick sections were immunohistochemically stained (streptavidin-biotin-peroxidase) with a monoclonal mouse anti human Von Willebrandt factor, which labels the endothelial cells (Dako, Denmark).

The microvessel density was assessed by selecting at 200 × magnification (20 objective and 10 ocular) the more immunolabelled areas, located in or under the gingival epithelium using a microscope (Nikon Eclipse E-600, Tokyo, Japan) coupled with a video camera (JVC TK-C1380E Tokyo, Japan). At least 20 areas were selected for each sample. The images were then captured at higher magnification (400 ×), and manual outlining of the vessels was performed; areas, perimeters and number of vessels per square millimetre were calculated, using an image analysis system (MONO, Imagine e Computer System, Milan).

Each immunolabelled endothelial cell separate from adjacent microvessels was counted as a single microvessel, according to standards of evaluation (Weidner *et al*., 1993). Data obtained expressed as mean and standard deviation were correlated with histological grades of dysplasias and carcinomas, by analysis of variance.

RESULTS

The count of immunolabelled vessels per 0.304 mm^2 on image analysis showed a progressive increase in number of vessels proceeding from normal gingivas, to dysplasias, to carcinomas. A decrease in the area and perimeter of vessels was observed in carcinomas as compared with dysplasias and normal gingivas. These differences were not statistically significant (Table I).

DISCUSSION

In human medicine, some clinical studies have demonstrated that 10–20% of oral dysplastic lesions progress to carcinoma and the risk of neoplastic transformation is 43% for severe dysplasia (Pazouki *et al*., 1997).

In this study a progressive increase in the number of vessels was observed from normal tissues, to dysplasias to carcinomas, suggesting an implication of angiogenesis in neoplastic progression also for oral cavity lesions of the dog. These results are in agreement with previous studies, in which the so-called "angiogenic switch", by which

TABLE I
Evaluation of angiogenesis by morphometric analysis of blood vessels in normal and pathological tissues

	Normal gingiva	Medium dysplasia	Severe dysplasia	Carcinoma	*p* value
Number of vessels	20	26	29	36	0.016
Area (mm^2)	194 ± 140	160 ± 135	140 ± 90	65 ± 52	0.08
Perimeter	46 ± 30	45 ± 26	40 ± 19	29 ± 18	0.02

a cellular phenotype commutes and becomes able to activate angiogenesis, is fundamental for the acquisition, by neoplastic cells, of features of malignity such as fast growth and metastatic capability.

The decrease in the areas and perimeters of vessels in severe dysplasias and carcinomas reflects an increased angiogenic rhythm, which can give rise to numerous, but small and often abnormal, new vessels.

In conclusion, angiogenesis may be involved in the neoplastic progression of gingival lesions in dogs, and microvessel density and vascular parameters may be sensitive prognostic parameters.

REFERENCES

Dvorak, H.F., Brown, L.F., Detmar, M. and Dvorak, A.M., 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hypermeability, and angiogenesis. *American Journal of Pathology*, **146**, 1029–1039

Folkman, J., Watson, K. and Ingber, D., 1989. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature*, **339**, 58–61

Folkman, J., 1990. What is the evidence that tumors are angiogenesis-dependent? *Journal of the National Cancer Institute*, **82**, 4–6

Fox, S.B., Gatter, K.C. and Harris, A.L., 1996. Tumour angiogenesis. *Journal of Pathology*, **179**, 232–237

Hanahan, D. and Folkman, J., 1996. Patterns and emerging mechanism of the angiogenic switch during tumorigenesis. *Cell*, **86**, 353–364

Maiolino, P., De Vico, G. and Restucci, B., 2000. Expression of vascular endothelial growth factor in basal cell tumours and in squamous cell carcinomas of canine skin. *Journal of Comparative Pathology*, **123**, 141–145

Maiolino, P., Papparella, S., Restucci, B. and De Vico, G., 2001. Angiogenesisi in squamous cell carcinomas of canine skin: an immunohistochemical and quantitative analysis. *Journal of Comparative Pathology*, **125**, 117–121

Pazouki, S., Chrisholm, D.M. and Adi, M.M., 1997. The association between tumour progression and vascularity in the oral mucosa. *Journal of Pathology*, **183**, 39–43

Pindborg, J.J., 1997. Histological typing of cancer and precancer of the oral mucosa. In: *World Health Organization International Histological Classification of Tumours* II, ed. Restucci, B., De Vico, G. and Maiolino, P., 2000. Evaluation of angiogenesis in canine mammary tumors by quantitative platelet endothelial cell adhesion molecule immunohistochemistry. *Veterinary Pathology*, **37**, 297–301

Restucci, B., Papparella, S., Maiolino, P. and De Vico, G., 2002. Expression of vascular endothelial growth factor in canine mammary tumors. *Veterinary Pathology*, **39**, 488–493

Vartanian, R.K. and Weidner, N., 1995. Endothelial cell proliferation in prostatic carcinoma and prostatic hyperplasia: correlation with Gleason's score, microvessel density, and epithelial cell proliferation. *Laboratory Investigation*, **73**, 844–850

Weidner, N., 1998. Tumoral vascularity as a prognostic factor in cancer patients: the evidence continues to grow. *Journal of Pathology*, **184**, 119–122

Veterinary Research Communications, 28 (2004) 303–306

Transmissible Spongiform Encephalopathy (TSE): Vaccinal Approach Using the Hamster Model

C. Pollera[1]*, M. Caramelli[2], M.L. Giannino[1], P.A. Martino[1], M. Puricelli[1], C. Casalone[2], P. Gazzuola[2] and G. Poli [1]

[1] *Department of Veterinary Pathology, Hygiene and Public Health, Microbiology and Immunology Unit, Faculty of Veterinary Medicine, University of Milan, Italy;* [2] *Centro per lo Studio e le Ricerche sulle Encefalopatie Animali e Neuropatologie Comparate, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle D'Aosta*

* *Correspondence: Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Microbiologia e Immunologia, via Celoria 10, 20133 Milan, Italy*

E-mail: claudia.pollera@unimi.it

Keywords: hamster, immunohistopathology, prion diseases, vaccine prophylaxis

Abbreviations: BSE, bovine spongiform encephalopathies; CNS, central nervous system; i.c., intracerebral; i.p., intra-peritoneal; vCJD, new variant of Creutzfeldt Jacob Disease; PrP, prion protein; PrPres, protease resistant prion protein

INTRODUCTION

Transmissible Spongiform Encephalopathies (TSE) are a group of disorders of the CNS that alarmed the scientific community especially because of the strictly correlated recent epidemics of BSE in cattle and vCJD in humans. The possible transmission of the disease from animal to man, the inability of the detection of the pathologic form of the prion protein (PrPres) before the appearance of symptoms and the lack of effective therapies against the infection make the development of prophylactic measures against TSE urgent, even if these are not easy to achieve due to the very low immunogenicity of the prion protein (Hill *et al*., 1997; Mabbott *et al*., 2001). Encouraging results have been obtained in the prevention of Alzheimer's disease and experimental scrapie in murine models using repeated injections of synthetic peptides or recombinant proteins: therefore, it seems to be possible to bypass the immune tolerance toward self proteins by using proper immunization protocols (Schenk *et al*., 1999; Sigurdsson *et al*., 2002). In this work we investigated whether active immunization of hamsters using synthetic peptides that reproduce different parts of the sequence of the homologous PrP and a fragment of human PrP can prevent or influence the evolution of the experimental prion disease.

MATERIALS AND METHODS

80 Golden Syrian Hamster (Charles River Italia S.P.A.) were used. The animals were randomly divided into five vaccine groups, respectively immunized with: heterologous

synthetic peptide covering the 82–146 aminoacid residuals of the human prion protein and four synthetic peptides (A, B, C and D) homologous to hamster prion protein. Peptides B, C and D are partially overlapping and cover the 105–128 (B) and 119–146 (C) aminoacid residuals of the N-terminal of the protein and the 142–179 residuals of the central region (D). Peptide A covers the 198–231 residuals of the C-terminal of the protein. The peptides were conjugated to KLH (Key Limpet Hemocyanin). The first vaccination and two boosters (at 10 day intervals) were performed one month before challenge. The first group of animals was infected by the intra-cerebral route with 10^3 LD_{50}/30 μl and the second group by the intra-peritoneal route with 10^5 LD_{50}/500 μl of 263 K hamster-adapted scrapie strain. Five boosters with the same antigens were given at 20 day intervals after the challenge. The brains of the animals that died during experimentation were removed and cut midsagittally; one cerebral hemisphere was kept in 20 μl of 10% formalin for histopathological and immuno-histochemical analysis. The presence and spread of typical neurological lesions and PrPres deposits in the brain were evaluated using hematoxylin-eosin staining, the deposits of pathologic prion protein was detected using a rabbit polyclonal antibody, anti PrP (kindly provided by the Health Ministry) and revealed using a peroxidase labelled anti-rabbit serum. The remaining cerebral hemisphere was kept at $-80°C$ for Western blotting analysis.

RESULTS

The animals immunized and infected by the intra-cerebral route died at the same time, or even before, the positive controls (Table I). The typical histopathological scrapie lesions were more evident in the immunized groups as compared with the controls: the medulla is the encephalic district in which the lesions appear more diffuse for all experimental groups. The immuno-histochemical analysis showed a slightly smaller amount of pathological protein only for animals immunized with the heterologous peptide PrP 82–146. The distribution of PrPres appeared quite uniform in the different encephalic districts, with less involvement of the hippocampus.

Differences in survival curves were observed in immunized and intra-peritoneally infected animals; hamsters immunized with peptides PrP 119–146 and PrP 142–179 survived 25 days longer than the controls (Table I) with no significant differences between them (p values 0.2517 and 0.2867, respectively). The amount of pathological protein in the brain was lower than for controls and was generally proportional to the extension of the histopathological lesions, even some exceptions were observed.

DISCUSSION

With the aim of explaining the early mortality of the i.-c. infected immunized animals as compared to the controls we hypothesized that an auto-immune response was

TABLE I
Survival time for immunized and infected animals (by intra-cerebral and intra-peritoneal route)

Group S		Peptides	Survival range	Mean	Δ
1		Non-immunized controls	99–132	112 ± 13.13	
2		(A) PrP 198–231	99–109	102.16 ± 4.79	−9.84
3	I.C. infection 263 K 10^3 LD_{50}	(B) PrP 105–128	96–126	112.8 ± 19.52	+0.8
4		(C) PrP 119–146	103–124	110 ± 7.7	−2
5		(D) PrP 142–179	96–109	100.83 ± 5.41	−11.17
6		(E) PrP 82–146	96–100	98.16 ± 1.47	−13.84*
7		Non-immunized controls	111–171	139.85 ± 24.53	
8		(A) PrP 198–231	98–216	150.83 ± 40.81	+10.98
9	I.P. infection 263 K 10^5 LD_{50}	(B) PrP 105–128	109–171	157.83 ± 49.28	+17.98
10		(C) PrP 119–146	112–171	165.16 ± 43.81	+25.66
11		(D) PrP 142–179	110–214	165.5 ± 54.88	+25.65
12		(E) PrP 82–146	111–157	137 ± 18.03	+2.85

*$p = 0.040$.

stimulated. We will verify this hypothesis by looking for complement fractions in CNS. In fact, autoimmune encephalitis occurred during clinical immunization trials with β-amyloid performed on Alzheimer's disease patients; for this reason, the trials were interrupted. We can conclude that the immunization can only induce a certain rate of resistance to experimental infection if the infection is performed via the intra-peritoneal route, which is more comparable to natural infection. The more effective immunogens in this experiment comprise the prion protein sequence probably involved in the PrPres conversion process. Immunization performed using the described protocols is to be considered protective as it can delay the onset of the symptoms and the death of the animals, but it cannot stop the disease.

ACKNOWLEDGEMENTS

This research was supported by grants from the Italian Ministry of Health, Strategic Project 2001

REFERENCES

Hill, A.F., Desbrulais, M., Joiner, S., Sidle, K.C.L., Gowland, I. and Collinge, J., 1997. The same prion protein cause vCJD and BSE, *Nature*, **389**, 448–450

Mabbott, N.A. and Bruce, M.E., 2001. The immunobiology of TSE diseases, *Journal of General Virology*, **82**, 2307–2318

Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yednock, T., Games, D. and Seubert, P., 1999. Immunization with amyloid-beta attenuates Alzheimer disease-like pathology in the PDAPP mouse, *Nature*, **400**, 173–177

Sigurdsson E.M. Brown, D.R., Daniels, M., Kascsak, R.J., Kascsak, R., Carp, R., Meeker, H.C., Frangione, B. and Wisniewski, T. 2002. Immunization delays the onset of prion disease in mice, *American Journal of Pathology*, **161**(1), 13–17

Veterinary Research Communications, 28 (2004) 307–310

In vivo Model for the Evaluation of Molecules Active Towards Transmissible Spongiform Encephalopathies

W. Ponti[1]*, M. Sala[2], C. Pollera[1], D. Braida [2], G. Poli[1] and S. Bareggi[2]
[1] *Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Microbiologia e Immunologia;* [2] *Dipartimento di Farmacologia Medica, Chemioterapia e Tossicologia, Milano. Centro di Eccellenza per le Malattie Neurodegenerative*
* *Correspondence: Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Microbiologia e Immunologia. Via Celoria 10. 20133 Milan, Italy*
E-mail: wilma.ponti@unimi.it

Keywords: anti-prionic therapy, clioquinol, general behaviour observations, hamster, *in vivo* models

Abbreviations: CNS, central nervous system; GBO, general behaviour observations; i.c., intra-cerebral; i.p., intra-peritoneal; PrP^{res}, protease resistant prion protein; TSE, transmissible spongiform encephalopathies

INTRODUCTION

The identification of suitable therapies against TSE (Transmissible Spongiform Encephalopathies) is a pressing problem to address. Different anti-prionic compounds have been shown to be effective *in vitro*, and some of the molecules tested are able to prolong the incubation period of the disease in experimentally infected animals (Brown, 2002). Alzheimer's Disease and prion diseases share a number of common features: both are characterized by the generation of misfolded proteins (PrP^{res} in prion disease), causing amyloid deposits in the CNS. The capability of Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline) to dissolve amyloid plaques in post-mortem brain tissue from Alzheimer's patients, and also to dissolve Alzheimer-like plaques in transgenic mice has recently been shown (Melov, 2002). Clioquinol acts as a zinc and copper chelator: the interaction with metals seems to be involved in the deposition and stabilization of amyloid plaques, therefore chelating agents can interfere with abnormal protein deposition by preventing metal/A-β amyloid interaction, (Cuajungco *et al*., 2000). Since copper and zinc binding is probably also involved in PrP^{res} conversion, we have tested the anti-prionic activity of Clioquinol on hamsters experimentally infected with scrapie.

MATERIALS AND METHODS

Different groups of Golden Syrian hamsters were infected either using the intra-cerebral (i.c.) or intra-peritoneal (i.p.) route, with a suspension of pooled brains (prion

titer of the inoculum = 10^3 LD_{50}/30 µl and 10^5 LD_{50}/500 µl respectively), derived from hamsters sacrificed at the final stage of experimental infection with the 263K scrapie strain. These animals were orally treated with 7.5 mg/kg/day of Clioquinol (Enteroviorformio®) dissolved in drinking water. One group of non-infected hamsters was also treated with Clioquinol (control of chronic toxicity). The animals were housed in single cages (IVC, Tecniplast) supplied with HEPA filtered air through a self-closing valve. Experimental procedures followed the EU International Guidelines for animal experimentation.

The procedures for evaluation of the anti-prionic activity of Clioquinol include General Behaviour Observations (GBO), which consists of analysis of passive avoidance and spontaneous motor activity (Braida *et al*., 2000; Sala *et al*., 1997), performed twice (61–65 days and 73 days post-infection for i.c.- and 150 days post-infection for i.p.-infected hamsters).

Passive avoidance: an apparatus consisting of a box divided by a wall into two compartments (one of which is dark) of the same size, in which the floor has a grid of steel rods, was used. Retention of the avoidance response was tested 24 h after the electric shock trial (2 mA/3 s). The latency of re-entry into the dark compartment was recorded up to a maximum of 180 s.

Spontaneous motor activity: this parameter was evaluated in an activity cage (U.Basile, Italy), placed in a sound-attenuating room. The cage was fitted with two parallel horizontal infrared beams located 2 cm from the floor. Cumulative horizontal movement counts were recorded for 10 min.

RESULTS

Animals chronically treated with Clioquinol showed no toxicity symptoms until the end of the experiment.

I.c. scrapie-infected animals treated with Clioquinol survived only 5.5 days longer than untreated controls. For Clioquinol-treated i.p. infected animals survival ($D = 40.7$ days) was significantly ($p = 0.0347$) longer than for untreated controls.

The results of GBO are shown in Fig. 1. Scrapie i.c.-infected hamsters showed a strong impairment of mnemonic function and a slight, but not significant, reduction of spontaneous motor activity at 63 days post-infection. No change in these parameters was recorded 73 days post-infection. Treatment with Clioquinol only produced a slight improvement in mnemonic function. Scrapie i.c.-infected hamsters showed a strong impairment of memory function and a moderate reduction of spontaneous motor activity at 150 days post-infection. Treatment with Clioquinol produced a slight, but not significant, improvement in mnemonic function, while it completely restored the spontaneous motor activity.

DISCUSSION

The early memory impairment observed in scrapie i.c.-infected animals suggests that the developing neuronal damage is in brain areas associated with learning. Therefore,

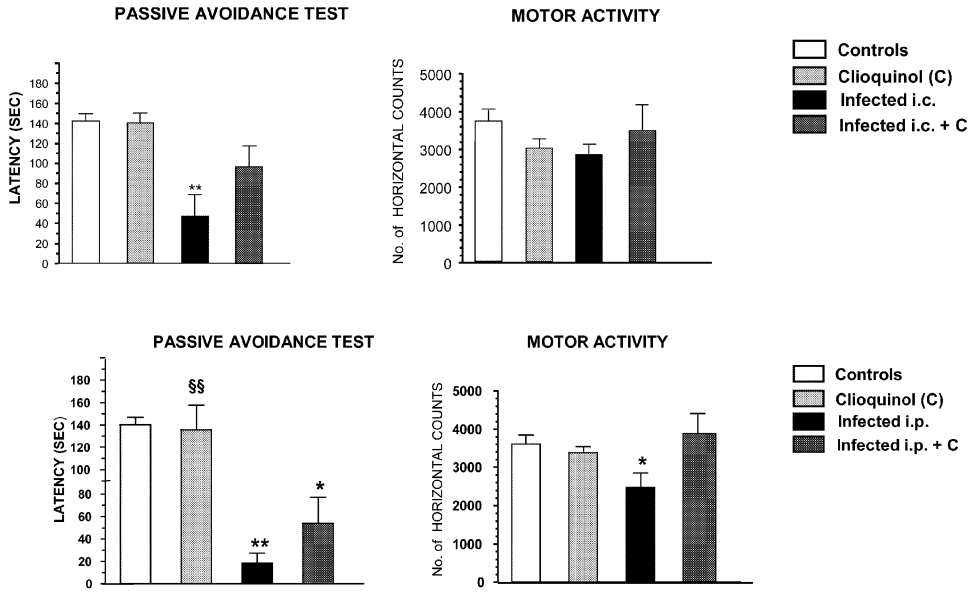


Figure 1. Result of general behaviour observation in animals infected by the i.c. route (upper graphs) and by the i.p. route (lower graphs). $p < 0.05$; $^{**}p < 0.01$ vs controls; $^{\S\S}p < 0.01$ vs infected

the evaluation of memory deficit can be a useful tool in the early detection of infection before development of the classical clinical signs.

The slight delay in memory and motor activity detriment and the improvement of motor activity observed for i.p.-scrapie infected hamsters chronically treated with Clioquinol, as well the significant elongation of the survival curve, suggests a possible therapeutic effect of the drug. The i.p. route of scrapie infection is closer to the natural infection, which is assumed to happen through the intake of infected food. These results suggest that Clioquinol can interfere with the development of the disease, as the GBO indicate. Therefore, this antibiotic possesses some of the ideal properties for a candidate agent against TSE, also because its low toxicity can allow experimentation into use of higher doses of the drug, which may be more active.

ACKNOWLEDGEMENTS

This research was supported by grants from the Italian Ministry of University and Scientific and Technological Research (Cofin 2002) and from the CARIPLO Foundation (2001).

REFERENCES

Braida, D. Pozzi, M. and Sala, M., 2000. CP 55,940 protects against ischemia-induced electroencephalographic flattening and hyperlocomotion in Mongolian gerbils. *Neuroscience Letters*, **296**(2–3), 69–72

Brown P., 2002. Drug therapy in human and experimental transmissible spongiform encephalopathy. *Neurology*. **58**(12), 1720–1725

Cuajungco, M.P., Faget, K.Y., Huang, X., Tanzi, R.E. and Bush, A.I., 2000. Metal chelation as a potential therapy for Alzheimer's disease. *Annals of the New York Academy of Sciences*, **920**, 292–304

Melov S., 2002, '… and C is for Clioquinol' – the AbetaCs of Alzheimer's disease. *Trends in Neurosciences*, **25**(3), 121–123

Sala, M., Leone, M.P., Lampugnani, P., Matturri, L. and Gori, E., 1997. Polydeoxyribonucleotide (defibrotide) protects against post-ischemic behavioral, electroencephalographic and neuronal damage in the gerbil. *European Journal of Pharmacology*, **328**(2–3), 143–152

Veterinary Research Communications, 28 (2004) 311–313

Principal Endoparasitoses of Domestic Cats in Sardinia

M. Porqueddu [1], A. Scala[1]* and V. Tilocca[2]
[1] *Department of Animal Biology – Section Parasitology – Faculty of Veterinary, University of Sassari, 07100 Sassari, Italy;* [2] *Professional Veterinary, 07100 Sassari, Italy*
* *Correspondence: Dipartimento di Biologia Animale, Sezione di Parassitologia – Facoltà di Medicina Veterinaria, Università di Sassari, Via Vienna, 2-07100 Sassari, Italy*
E-mail: scala@uniss.it

Keywords: cat, epidemiology, endoparasites, digestive and broncho-pulmonary apparata

INTRODUCTION

With few exceptions, the parasitological profile of cats has been poorly researched. This is probably because of the objective difficulties experienced during the sampling stage, such as blood sampling or exact identification of faecal samples taken from subjects living in colonies. For these reasons, and considering that no research has been conducted on this topic in Sardinia, except for the paper by Arru and Lai on Ancylostomosis back in 1965, we decided to report here the results of an epidemiological survey conducted on our island. The aim was to assess the main parameters of the principal parasitoses present, some of which are also of zoonosic interest (*Toxoplasma* spp., *Toxocara* spp., *Uncinaria* spp., *Dipylidium* spp.).

MATERIALS AND METHODS

160 cats aged between 2 months and 16 years belonging to different breeds, some of which lived exclusively in flats, and others more or less regularly outside, were coprologically monitored over the period between April 2000 to October 2001. In addition, IgG and IgM anti-*Toxoplasma gondii* were sought in the serum of 54 subjects using indirect immunofluorescence (IFI).

The faecal samples were first examined macroscopically to check for the possible presence of proglottides which were observed without any staining and/or by colouring with hydrochloric carmine and which were then isolated and classified. The taxonomic keys normally used were those indicated by Khalil *et al.* (1994). The method used in the subsequent copromicroscopic examinations is one that provides for sedimentation and flotation with resolution of specific gravity 1450. 54 of these samples were additionally processed to check for the presence of larvae of *Aelurostrongylus abstrusus* using Baermann's methodology. A necroscopic examination was carried out on 23 subjects which had died of natural causes (or because they

were run over by motor vehicles) with the aim of isolating and identifying the parasitic agents present at the gastro-intestinal, broncho-pulmonary and cardiac level. The propagation forms found and the samples of parasites isolated on autopsy were classified according to the morphometric keys proposed by Sloss and Kemp (1985) and Thienpont *et al.* (1986).

RESULTS AND DISCUSSION

A serum prevalence of 37% of IgG and no positive results for IgM were found for *Toxoplasma gondii*; and no oocysts of this protozoan were found in the faeces. It is thus evident that we were faced exclusively with the chronic stages of this infection, and this confirms that the role of the cat in the spread of this parasitosis is marginal and/or exclusively linked to very young subjects and/or those in bad immunitary conditions. Furthermore, the serological data do not differ too much from those noted in other national districts (33% Tenter *et al.*, 2000) or international data (42% Rhode Island, U.S.A. – De Feo *et al.*, 2002; 26.3% Sao Paolo, Brasil – Silva *et al.*, 2002). Table I shows the results obtained both from the coprological examination and from autopsies. Total percentage of infestations and/or mono-specific infections were 26.9%.

The stratification of results obtained for cats according to sex did not show any

TABLE I
Results obtained both from the coprological examination and from autopsies

	Coprological exam		
Endoparasite	Macroscopic	Microscopic	Autopsy
Protozoa			
Isospora felis	/	17 (10.6%)	1 (4.3%)
Isospora rivolta	/	1 (0.6%)	0
Nematoda			
Ancylostoma tubaeforme	/	5 (3.1%)	1 (4.3%)
Aelurostrongylus abstrusus	/	1 (0.6%) 4 (7.4%)*	5 (21.7%)
Toxocara cati	/	9 (5.6%)	3 (13%)
Cestoda			
Dipylidium caninum	14 (8.8%)	2 (1.2%)	3 (13%)
Diplopylidium spp.	0	0	5 (21.7%)
Hydatigera taeniaeformis	3 (1.9%)	0	5 (21.7%)
Mesocestoides lineatus	0	0	3 (13%)

*Data obtained using Baermann's methodology for 54 samples.

significant difference with regard to the total number of parasitoses found ($p = 0.284$); whereas the stratification of results according to the living environment of the cats showed important variations in the rates of predominance: cats living in flats 15%; cats living outdoors 40%; cats living in a mixed environment 37.7% ($p = 0.003$). This last piece of data is not justified if antiparasitic treatments are considered, which we found to be evenly practised in the three categories examined ($p = 0.79$). It is thus evident that living environment is one of the most important factors in the onset and persistence of the infestation of cats on the island.

In general the results obtained show that cats in Sardinia are in a less serious condition with regard to intestinal parasitoses as compared to data in other regions of Italy (specifically northern), where the prevalence of *Toxocara* spp. reached almost 50%: 10% for *A. tubaeforme* and 41% for *D. caninum* (Poglayen *et al.*, 1985). However, infestation by *A. abstrusus*, a parasitosis that is generally underestimated, is another matter altogether.

In the present research, we have shown that normal copromicroscopic analyses carried out on live animals can generally only reveal the presence of this nematode in a limited number of cats, in contrast with results obtained using Baermann's methodology or an autoptic examination. It is clear, then, that if parasitoses are present in over 20% of autopsies carried out on cats, it is to be hoped that any routine examination carried out in veterinary surgeries would include Baermann's methodology, bearing in mind the pathogenic power generally attributed to this nematode (Marcato, 1988). Moreover, research unfortunately confirms, once again, the limits of parasitological analyses, particularly when confronted with Cestoda, which were found for 39.1% of cats undergoing an autopsy, as compared to 10.6% of those analysed copromicroscopically ($p < 0.001$). In no case was there evidence of Filariid nematodes in the cardiac cavities or pulmonary arteries.

REFERENCES

Arru, E. and Lai, M., 1965. *Ancylostoma tubaeforme* (Zender, 1800) in gatti selvatici e domestici della Sardegna. *Studi sassaresi*, **4–5**, 517–524

DeFeo, M.L., Dubey, J.P., Mather, T.N. and Rhodes, R.C., 2002. Epidemiologic investigation of seroprevalence of antibodies to *Toxoplasma gondii* in cats and rodents. *American Journal Veterinary Research*, **63**(12), 1714–1717.

Khalil, L.F., Jones, A. and Bray, R.A., 1994. *Keys to the Cestode Parasites of Vertebrates* (CRC Press: Florida)

Marcato, P.S., 1988. *Patologia respiratoria animale* (Ed agricole, Bologna)

Poglayen, G., Traldi, G., Capelli, G. and Genchi, C., 1985. Fauna parassitaria gastro-intestinale del gatto nelle Città di Bologna, Firenze e Milano. *Parassitologia*, **27**, 297–302

Silva, J.C.R., Gennai, S.M., Ragozzo, A.M.A., Amajones, V.R., Magnabosco, C., Yai, L.E.O. and Dubey J.P., 2002. Prevalence of *Toxoplasma gondii* antibodies in sera of domestic cats from Guarulhos and San Paulo, Brazil. *Journal of Parasitology*, **88**(2), 419–420

Sloss, M.G. and Kemp, R.L., 1985. *Parassiti in medicina veterinaria* (Edi. Ermes, Milano)

Thienpont, D., Rochette, F. and Vanparijs, O.F.J., 1986. *L'esame coprologico nella diagnosi delle elmintiasi*, seconda edizione (Janssen Research Foundation, Beerse, Belgio)

Tenter, A.M., Heckeroth, A.R. and Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans, *International Journal for Parasitology,* **30**(12–13), 1217–1258

Veterinary Research Communications, 28 (2004) 315–317

Mycobacterioses: Emerging Pathologies in Aquarium Fish

M. Prearo[1]*, R.G. Zanoni[3], B. Campo Dall'Orto[1], E. Pavoletti[1], D. Florio[3], V. Penati[4] and C. Ghittino[2]
[1] *Fish Disease Laboratory, IZS – State Veterinary Institute of Piedmont, Liguria and Aosta Valley, Turin, Italy;* [2] *Fish Disease Laboratory, IZS – State Veterinary Institute of Umbria and Marche, Perugia, Italy;* [3] *Fish Disease Laboratory, Dept. of Veterinary Public Health and Animal Pathology, School of Veterinary Medicine, Ozzano Emilia (Bologna), Italy;* [4] *Villa Marelli Institute, Niguarda-Ca' Granda Hospital, Milan, Italy*
* *Correspondence: Laboratorio di Ittiopatologia e Acquacoltura, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, 10154 Torino – Italy*
E-mail: marino.prearo@izsto.it

Keywords: fish tuberculosis, *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Mycobacterium marinum*, ornamental fish

INTRODUCTION

Mycobacterioses, also known as Tuberculosis, are the most common chronic bacterial diseases in ornamental fish, affecting both temperate and tropical species both in freshwater and the marine environment (Ghittino, 1985; Bozzetta *et al*., 1995; Chinabut, 1998; Prearo *et al*., 2002). Moreover, they may be found both in wild fish and those cultured for human consumption (Colorni, 1992; Dos Santos *et al*., 2002). Besides inducing mortality in fish, they represent a potential hazard for man, being included among zoonoses (Ghittino and Bozzetta, 1994). Mycobacterioses are caused by ubiquitous bacteria that are highly resistant in the aquatic environment and are difficult to control. *Mycobacterium fortuitum*, *M. marinum* and *M. chelonae* are the most commonly isolated species. They are all capable of inducing human cutaneous infections, with development of nodular lesions that are generally localized in the limbs (Ghittino, 1985). In fish, symptoms appear late, are non-specific and include slow growth, lethargy, anorexia and starvation. Lesions in the skin and typical whitish nodules in the viscera may be detected at necropsy (Ghittino, 1992). Therapy is rather difficult, both for the fact that early diagnosis is seldom achieved and for the lack of effective drugs (Ghittino, 1985).

In the present work, the results from an 18 month survey on cultured and wild ornamental fish are reported, with the aim of improving knowledge on the distribution of Mycobacterioses in Italy.

MATERIALS AND METHODS

312 aquarium fish, both of domestic and foreign origin, were sampled by importation and distribution centers and by private centers, over a period from July 2001 to

February 2003. 266 freshwater and 46 marine fish were subjected to necropsy and, when nodular lesions were observed, Ziehl-Neelsen stained imprints were performed. Portions of liver, spleen and kidney were fixed in a 10% formalin solution, processed and stained with hematoxylin-eosin and Ziehl-Neelsen for histological examination. A specific bacteriological examination was carried out on all fish. The entire specimen, in the case of small fish, or the viscera, for larger fish, were minced, diluted in deionized sterile water, homogenized and filtered. This solution was decontaminated with HPC (1:1), centrifuged and the pellet obtained was inoculated into selective media (Loewenstein-Jensen and Stonebrink), and incubated at 30°C until colonies developed. Colonies that were positive at Ziehl-Neelsen were subcultured to perform biochemical identification. The tests utilized were nitrates reduction, urease, growth on 5% NaCl medium, growth at 37°C, growth on McConkey agar without crystal violet, niacin, Tween hydrolysis, arylsulfatase, cold catalase and catalase at 68°C for 20 min.

RESULTS

135 samples, out of 312 analyzed, were found to be positive for mycobacteria on bacteriological examination (45%). 75 of these had already been found positive by imprint (55%). Among these clear macroscopic findings of Tuberculosis were observed only for 38 fish (50%). 34 specimens with tubercular lesions belonged to freshwater species and 4 to marine species. Exophthalmos and keratitis were observed, but not skin lesions reported in literature (Ghittino, 1985; Chinabut, 1998). Visceral nodules had a size of 0.5–12 mm and were variable in number. 17 out of the remaining 37 fish without tubercular lesions but identified as being positive by imprint, were positive on histological examination. Granulomas, at different stages of development, were found in the liver, spleen and kidney. These were made of epithelioid cells containing acid-fast bacteria, surrounded by a thick connective capsule. Out of 135 isolates of mycobacteria, 109 were phenotypically identified as *Mycobacterium fortuitum*, 16 as *M. chelonae*, 5 as *M. marinum*, 3 as *M. terrae* and 2 as *M. gordonae*. Mixed infections, caused by different mycobacterial species, were never observed in the same fish sample. The most common freshwater fish affected by Mycobacterioses were the gold fish (*Carassius auratus*), the Siamese fighting fish (*Betta splendens*), *Colisa lalia* and *Barbus tetrazona*. Among marine fish the most affected species was found to be *Zebrasoma veliferum*.

CONCLUSIONS

Our results confirm that Mycobacterioses are widely spread among ornamental fish in Italy and may be considered as one of the main bacterial diseases. For this reason, these should always be suspected in weak and stressed fish. Unlike what is reported

in the literature, where the most frequent mycobacterial species in freshwater fish is considered to be *Mycobacterium marinum* (Ghittino, 1985; Chinabut, 1998), we found a higher prevalence of *M. fortuitum* and *M. chelonae*. The exact role played by *M. terrae* and *M. gordonae* as fish pathogens is still unknown, since these mycobacteria were isolated from fish without macroscopic or microscopic signs of disease. Considering the increasing importance of aquarium fish as pet animals and the lack of specific laws on this topic, a focused study into the health risks due to mycobacteria in ornamental fish is recommended.

REFERENCES

Bozzetta, E., Prearo, M., Penati, V., Pungkachonboon, T. and Ghittino, C., 1995. Isolamento e tipizzazione di micobatteri in pesci tropicali d'allevamento. *Bollettino Società Italiana di Patologia Ittica*, **16**, 13–21

Chinabut, S., 1998. Mycobacteriosis and Nocardiosis. In *Fish Diseases and Disorders*, Vol. 3, Ed. Woo P.T.K. and Bruno, D.W. CAB International Publishing, 319–340

Colorni, A., 1992. A systemic mycobacteriosis in the European sea bass *Dicentrarchus labrax* cultured in Eilat (Red Sea). *Israeli Journal Aquaculture*, **44**(3), 75–81

Dos Santos, N.M.S., Do Vale, A., Sousa M.J. and Silva, M.T., 2002. Mycobacterial infection in farmed turbot *Scophthalmus maximus*. *Disease Aquatic Organisms*, **52**, 87–91

Ghittino, C., 2002. Indagine sulla diffusione delle Micobatteriosi ittiche e sui relativi reperti anatomoistopatologici. *Tesi di Specializzazione in Allevamento, igiene, patologia delle specie acquatiche e controllo dei prodotti derivati*. Università degli Studi di Padova, 1–50

Ghittino, C. and Bozzetta, E., 1994. Profilassi delle zoonosi di origine ittica. *Medicina Veterinaria Preventiva*, **7**, 5–6

Ghittino, P., 1985. *Tecnologia e patologia in Acquacoltura*, Vol. II – Patologia. Bono Editore, Torino, Italy.

Prearo, M., Latini, M., Proietti, M., Mazzone, P., Campo Dall'Orto, B., Penati V. and Ghittino, C., 2002. Casi di micobatteriosi in pesci rossi d'acquario. *Bollettino Società Italiana di Patologia Ittica*, **33**, 30–43

Veterinary Research Communications, 28 (2004) 319–322

Antigenic Variability of Ovine Lentivirus Isolated in Italy

S. Rosati[1]*, M Profiti[1], E. Grego, M.L. Carrozza[2], M. Mazzei[3] and P. Bandecchi[3]
[1] *Dipartimento di Produzioni Animali Epidemiologia ed Ecologia, Università di Torino;* [2] *Scuola Normale Superiore, Pisa;* [3] *Dipartimento di Patologia Animale, Università di Pisa*
* *Correspondence: Dipartimento di Produzioni Animali, Epidemiologia ed Ecologia-Facoltà di Medicina veterinaria Via Leonardo da Vinci, 44 10095 Grugliasco (TO), Italy*
e-mail: sergio.rosati@unito.it

Keywords: small ruminant lentiviruses, structural proteins, antigenic variability

INTRODUCTION

Small ruminant lentiviruses (SRLV) are a heterogeneous group of viruses causing persistent infection and chronic debilitating diseases in sheep and goats worldwide. Economic losses are related to the drop in milk production due to progressive pneumonia, lameness, and mastitis. Phylogenetic studies have been carried out using ovine or caprine viral strains obtained from different geographical regions and recent data suggest that a differentiation of SRLV based on host species origin may not be accurate (Zanoni, 1998). This is consistent with the identification of Maedi-Visna Virus (MVV)-like strains in goat and Caprine Arthritis-Encephalitis Virus (CAEV)-like strain in sheep. Recently a new cluster of ovine lentivirus isolates, related to CAEV has been described in France and Italy (Leroux *et al*., 1997; Grego *et al*., 2002). Phylogenetic studies have mainly been carried out using *pol* gene fragments, while limited information is available for gene fragments encoding major structural proteins involved in the antibody response and used for the development of serological tests. The use of a single strain-based immunoassay in detecting sheep and goat lentivirus infection is based on the finding that immunodominant regions are mainly cross-reactive among ovine isolates, as well as between ovine and caprine lentiviruses (Gogolewski *et al*., 1985). However, recent studies suggest that the heterogeneity of the immunodominant epitope of the capsid antigen may affect sensitivity of serological tests based on a single strain (Grego *et al*., 2002). In this study the *gag* gene of two genetically distinct ovine lentiviruses was characterized and epitope variability in the matrix protein (p16) and in the maior capsid antigen (p25) was evaluated.

MATERIAL AND METHODS

Two ovine lentiviruses isolated in Italy were used for this study. The former, It-561, has been previously classified in the MVV cluster while the latter, It-Pi1, has been

clustered in the new genotype named CAEV-like. Partial sequences of the *pol* and *gag* genes of the two strains have been previously determined (Grego *et al*., 2002). On the basis of the available sequences coding for the *pol* gene (GenBank accession number AY044811 and AY044815) and the highly conserved LTR region, specific primers were selected to amplify a 4 kb genomic fragment encompassing the entire *gag* gene of the two lentiviruses. Amplicons of the expected length were cloned into the pDRIVE vector and sequenced. The *gag* gene sequences of strain It561 and It-Pi1 were aligned with the consensus sequence of the SRLV sequences available in the GenBank database. Genetic distances were computed using MEGA (Kumar *et al*., 2001) and were used to construct a neighbour-joining tree (Saitou *et al*., 1987). Statistical support for the tree was evaluated by bootstrapping (Felsenstein, 1985).

Immunodominant regions of p25 have been previously characterized (Rosati *et al*., 1999), while relevant epitopes of p16 were determined in this study using recombinant subunits expressed in frame with glutathione S-transferase. Fusion proteins were purified using affinity chromatography and used in indirect ELISA to characterize the reactivity of sheep sera collected from different Italian flocks.

The nucleotide sequence data reported in this paper have been submitted to GenBank (Accession number AY265455 for It-561 and AY265456 for It-Pi1).

RESULTS

Phylogenetic analysis carried out on the *gag* gene confirmed that strain It-561 was genetically similar to MVV Icelandic prototype K1514 while strain It-Pi1 was more similar, although distinct from, the CAEV strain CO (Fig. 1). Two immunodominant regions of p25 (previous study) (Rosati *et al*., 1999) and p16 (this study) were identified in the two strains and a moderate variability in the aminoacid sequence was observed (see Table I). Ovine sera used in the present study mainly exhibited reactivity against the antigens derived from It-Pi1.

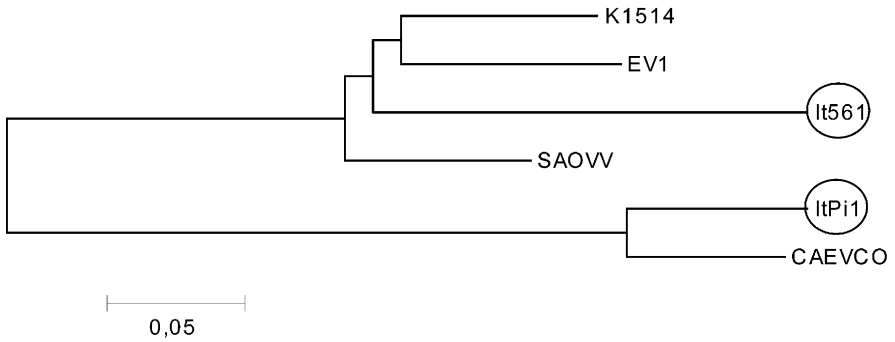


Figure 1. Phylogenetic analysis of the *gag* gene of two Italian ovine lentivirus isolates (circled)

TABLE I
Immunodominant linear epitopes of the matrix protein and major capsid antigen of CAEV-like (It-Pi1) and MVV-like (It-561) ovine lentivirus isolates

Strain	Matrix protein	Major capsid antigen
It-Pi1	KLLTPEESNKKDFMSL	GNRAQKELIQGKLNEEAERWRRNNPPPPA
It-561	-N-----TS-RE-A--	----------D-----V-Q---G-N

DISCUSSION

A previous phylogenetic study has been conducted in Italy on SRLV isolated in the last decade. Most ovine isolates (8 out of 9) belonged to the same cluster of It-Pi1 named CAEV-like. Thus, in Italy, many of the infected ovine population harbour viral strains which are different from those employed in the currently available diagnostic immunoassays. In fact, in different commercially available ELISAs, recombinant antigens have been developed from the classical ovine lentivirus genotype (i.e. SAOVV, EV1, ZZV1050) (Saman *et al*., 1999; Zanoni *et al*., 1991). This genotype is still circulating in our country, as demonstrated by isolation of strain It-561. The genetic variability of the field isolated raises a question regarding the validity of the single genotype-based serological test. This study clearly shows that immunodominant regions of two structural proteins are quite variable in the two ovine genotypes. A more accurate epitope characterization using overlapping fragments revealed a common or less variable N-terminal epitope and a distinct C-terminal epitope. Thus, the cross-reactivity between different genotypes is given by these and other conserved sequences. However, a subset of infected population may be partially reactive against common epitopes and not be detected using a diagnostic test based on the heterologous genotype. For CAEV infected goat flocks we observed that the homologous antigen is able to detect an additional 7% of seropositive animals as compared with heterologous antigen (Grego *et al*., 2002).

The systematic use of a single strain immunoassay in an infected population, followed by slaughtering of seropositive animals may select for "diagnostic test escape" viral mutants, thus interfering with eradication programs.

The recombinant subunits employed in the present study could be used in parallel to evaluate the genotype circulating in the flock under study and subsequently apply the homologous antigen for diagnostic purposes. Alternatively, a combination of antigens derived from the two genotypes in a single test may increase the sensitivity of serological diagnosis.

ACKNOWLEDGMENTS

This work was supported by the Italian MIUR.

REFERENCES

Felenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791

Gogolewski, R.P., Adams, D.S., McGuire, T.C., Bauks, K.L. and Cheevers, W.P., 1985. Antigenic cross-reactivity between caprine arthritis-encephalitis, visna and progressive pneumonia viruses involves all virion-associated proteins and glycoproteins. *Journal of General Virology*, **66**, 1233–1240

Grego, E., Profiti, M., Giammarioli, M., Giannino, L., Rutili, D., Woodall, C. and Rosati, S., 2002. Genetic heterogeneity of small ruminant lentiviruses involves immunodominant epitope of capsid antigen and affects sensititity of single-strain-based immunoassay. *Clinical and Diagnostic Laboratory Immunology*, **9**, 828–832

Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M., 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*, **17**, 1244–1245

Leroux, C., Chastang, J., Greenland, T. and Mornex, J.F., 1997. Genomic heterogeneity of small ruminant lentiviruses: existence of heterogeneous populations in sheep and of the same lentiviral genotypes in sheep and goats. *Archive of Virology*, **142**, 1125–1137

Rosati, S., Mannelli, A., Merlo, T. and Ponti, N., 1999. Characterization of the immunodominant cross-reacting epitope of visna maedi virus and caprine arthritis-encephalitis virus capsid antigen. *Virus Research*, **66**, 109–116

Saitou, N. and Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425

Saman, E., Van Eynde, G., Lujan, L., Extramiana, B., Harkiss, G., Tolari, F., Gonzales, L., Amorena, B., Watt, N. and Badiola, J., 1999. A new sensitive serological assay for the detection of lentivirus infections in small ruminant. *Clinical and Diagnostic Laboratory Immunology*, **6**, 734–740

Zanoni, R.G., 1998. Phylogenetic analysis of small ruminant lentiviruses. *Journal of General Virology*, **79**, 1951–1961

Zanoni, R.G., Nauta, I., Pauli, U. and Peterhans, E., 1991. Expression in *Escherichia coli* and sequencing of the coding region for the capsid protein of Dutch maedi-visna virus strain ZZV 1050: Application of recombinant protein in enzyme-linked immunosorbent assay for the detection of caprine and ovine lentiviruses. *Journal of Clinical Microbiology*, **29**, 1290–1294

Veterinary Research Communications, 28 (2004) 323–325

24-Hour Ambulatory Electrocardiography in the Dog

F. Birettoni, F. Porciello, F. Rueca and G. Fruganti
Department of Clinical Sciences – Section of Internal Medicine, Faculty of Veterinary Medicine, University of Perugia
**Correspondence: Dr. Francesco Birettoni-Dipartimento di Patologia, Diagnostica e Clinica Veterinaria – Sezione di Medicina Interna – Facoltà di Medicina Veterinaria, Università di Perugia, Via S. Costanzo 4, 06126 – Perugia, Italy*
E-mail: docbirrino2@libero.it

Keywords: arrhythmias, dog, electrocardiography, Holter, 24-hour ambulatory ECG

INTRODUCTION

Physical examination and standard electrocardiography have been used for several years to check the cardiac electrical activity and in particular to detect cardiac arrhythmias. Routine electrocardiography is the gold standard for the diagnosis of cardiac arrhythmias in practice. However, as a standard ECG lasts 1 or 2 min, that is to say 0.07–0.14% of 24 h, its sensitivity to cardiac arrhythmias is correlated with the frequency at which they occur. ECG sensitivity is high for continuous arrhythmias, such as atrial fibrillation in the dog, or very frequent arrhythmias, such as certain cases of ventricular premature complexes (VPCs), but is very low for detection of sporadic arrhythmias such as parossistic tachycardia. Furthermore, standard ECG requires the patient to be restrained in right lateral recumbency, influencing the autonomic nervous system and potentially inducing or suppressing cardiac arrhythmias (Doxey *et al*., 1985). In 1963 N.J. Holter proposed monitoring of cardiac rhythm for 24–48 h using a portable recorder to look for arrhythmias during the normal activity of the patient in order to increase the sensitivity of the ECG to cardiac arrhythmias. Nowadays 24-hour ambulatory electrocardiography (Holter monitoring) is widely used in human medicine, and less commonly in veterinary medicine, to detect cardiac arrhythmias in patients affected by syncope, sudden weakness or sudden dyspnoea, and to assess the efficacy of antiarrhythmic treatment. Furthermore, Holter monitoring can be useful for an early diagnosis of cardiac arrhythmias in particular dog breeds in which arrhythmogenic cardiomyopathies are inherited or where modifications of the cardiac rhythm are associated with extracardiac disease.

MATERIALS AND METHODS

Holter recordings were obtained using a Braemer DL 700 digital recorder (15.24–8.89–2.41 cm; 283 gr) that stored the data in a 10 MB flash card. We shaved

the thorax bilaterally over the middle and ventral third of the ribs, between the 5th and the 8th rib on the right side and between the 3rd and 7th rib on the left. We cleaned the skin using gauze with alcohol in order to remove sebum and dirt from the surface and to ensure a good contact between electrodes and the skin. We used self-adhesive electrocardiographic patches with 2.4 cm diameter. Each patch was covered with self-adhesive tape to avoid their displacement or detatchment. Seven electrodes were placed in order to obtain 3 leads with a V1, V3, V5 modified system (Gershwin, 1975; Doxey *et al*., 1985; Elie and Hoenig, 1995). Electrocardiographic patches and their wires were secured using a strip of self-adhesive tape that fixed the recorder between the scapulae on the withers region. The recorder was further secured to the patient using a 3M Vetrap™ elastic bandage that also covered the wires and the patches. Two loops of elastic bandage were crossed between the forelimbs to give more stability to the recorder and electrodes and to avoid them moving caudally and laterally. Each dog went back home with its owner who had to check the patient during recording to avoid damage to the recorder and to note all activity of the patient. The recording was then processed using the Holter Win P-V 5.40 Analyser installed on a notebook. The analysis was based on the general morphologic ECG features of the analyser but it was possible to modify and to upgrade them constantly before, during and after the automatic analysis. The tables with maximum, minimum and mean heart rate, supraventricular and ventricular ectopies and full disclosure of the recording were printed out for each patient.

We performed the Holter monitoring as an ancillary diagnostic test for 8 dogs of different breeds and sex, with an age range of 1 to 10 years and with a weight ranging from 5 to 72 kg. Each dog was conducted to the Section of Internal Medicine of the Pathology, Diagnostic and Veterinary Clinic Department of the Veterinary Faculty, University of Perugia for a cardiologic check up. We also performed a Holter recording in 3 clinically normal dogs as a control group.

RESULTS AND DISCUSSION

Each recording was obtained without any kind of restriction or of change in the daily activity of the patient. Holter recordings were clear enough to monitor the cardiac rhythm during the examination, even though in some cases they lasted less than 24 h, and to allow the analyser to work properly. It was possible to identify cardiac arrhythmias undiagnosed by physical examination or standard ECG and to have an electrocardiographic diagnosis in each case. Some of the arrhythmias we found were considered pathological and requiring proper antiarrhythmic treatment.

Physiological arrhythmias such as marked sinus bradycardia or sinus arrest, even lasting 3.5 s, or a brief period of sinus tachycardia, with the heart rate over 250 bpm, which can be considered the result of the autonomic nervous system influence (Davies *et al*., 1984) were detected, as well as definitely pathological arrhythmias: ventricular

and supraventicular ectopic beats and tachycardia, sick sinus syndrome, second degree AV block and paroxysmal atrial fibrillation.

REFERENCES

Fox, P.R., Sisson, D. and Moise, N.S., 1999. *Textbook of canine and feline cardiology*. WB Saunders, Philadelphia

Goodwin, J.K., 1998. Holter monitoring and cardiac event recording. *Veterinary Clinics of North America Small Animal Practice*, **28**(6), 1391–1407

Moise, N.S. and Defrancesco, T., 1995. Twenty-four hour ambulatory electrocardiography (Holter monitoring), in Kirk, "Current Veterinary Therapy XII Small Animal Practice". WB Saunders, Philadelphia

Porciello F, 2003. *Elettrocardiografia nel cane, gatto e cavallo*. Poletto Editore, Milano

Veterinary Research Communications, 28 (2004) 327–330

Plasma Thrombomodulin Levels in Dogs Naturally Infected with *Leishmania infantum*

P. Ciaramella *, L.Cortese, M. Corona, R. Ambrosio, A. Di Loria and A. Persechino
Department of Veterinary Clinical Science – Section of Internal Medicine – University of Naples Federico II, via Delpino, 1 – 80137 Naples, Italy.
** Correspondence: Dipartimento di Scienze Cliniche Veterinarie – Sezione di Clinica Medica – Facoltà di Medicina Veterinaria, via Delpino, 1– 80137 Napoli, Italy*
E-mail: paociara@unina.it

Keywords: dog, leishmaniasis, vasculitis, thrombomodulin, endothelium

Abbreviations: TM, thrombomodulin; IFAT, immunofluorescent antibody test; TP, total plasma proteins; ALT, alanine transaminase; PT, prothrombin time; APTT, activated partial thromboplastin time

INTRODUCTION

Plasma thrombomodulin (TM) is a membrane glycoprotein in the vascular endothelium which shows high affinity to thrombin. The thrombomodulin-thrombin complex induces coagulation by activation of protein C that inactivates the intrinsic pathway plasma factors V and VIII, finally blocking the formation of new thrombin. Plasma TM is mainly produced by endothelial cells and to a lesser extent by lymphocytes, neutrophils, monocytes, etc. Several studies performed, both in humans and laboratory animals, showed that TM levels were increased during haemostatic disorders (disseminated intravascular coagulation, thrombosis, etc) and during several diseases that induce endothelial damage (such as diabetes mellitus, lupus erythematosus and atopia), and renal and hepatic involvement, rickettsiosis, etc. The main characteristics of such diseases are micro and macroangiopathies due both to direct action of infective agents or to immune activity. The presence of vasculites has also been reported during leishmaniasis and it can be responsible for a very severe and often fatal evolution of the disease (Pumarola *et al*., 1991). Antigen-antibody immunocomplexes deposit in the endothelium inducing complement activation and inflammation. In canine leishmaniasis clinical signs such as renal failure, uveitis, arthritis, etc. are generally due to this autoimmune process. When these clinical signs are evident it is generally too late for a therapeutic approach, therefore, identification of markers for early detection of endothelial damage can be of great help to the veterinarian. The object of this study was to evaluate plasma TM concentrations in dogs affected by leishmaniasis at different grades of clinical involvement, in order to verify if plasma TM could also be utilized in dogs, as in humans, as a marker for endothelial injury. Moreover, the study of such a marker could lead to a better understanding of the mechanisms involved in this zoonosis.

MATERIALS AND METHODS

Plasma TM levels were measured in 38 animals: 24 dogs naturally infected by *Leismania infantum* (none of the animals had ever undergone any specific anti-Leishmania therapy) and 14 healthy dogs (control group). Sick dogs were divided into two groups on the basis of the clinical examination (Ciaramella *et al*., 1997). The first group comprised 10 oligo-symptomatic dogs while the second group comprised 14 symptomatic or markedly symptomatic dogs. The clinical diagnosis was always confirmed by direct observation of the protozoan in Giemsa stained fine needle aspirates of bone marrow and lymph nodes, and serologically using the immunofluorescent antibody test (IFAT). The threshold titre for positivity was 1/160. A full blood count and measurement of total plasma proteins (TP), serum protein electrophoresis, alanine transaminase (ALT), creatinine and urea serum levels were obtained for each dog. Prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen were also determined using a semi-automatic coagulometer. TM concentration in the plasma was measured using ELISA with a commercially available kit (American Diagnostic Inc., USA). Mean values with their standard deviations were calculated from the data obtained and were then statistically evaluated using one-way analysis of variance. Significant results were subjected to the Student–Newman–Keuls Multiple Comparisons Test.

RESULTS

The mean $\pm$ SD of plasma levels of thrombomodulin in healthy dogs was 2.51 ± 0.5 ng/ml, while in dogs with leishmaniasis it was 3.27 ± 0.8 ng/ml ($p < 0.01$): group I: 2.9 ± 0.4 ng/ml, group II: 3.63 ± 0.9 ng/ml ($p < 0.001$ vs control group and 0.05 vs group II). The results of coagulation tests were in the normal range (Table I). Normocytic and normochromic anaemia, slight thrombocytopenia, increased activities of serum ALT, increase in beta and gamma globulins and moderate hypoalbuminaemia were observed for the second group.

DISCUSSION

Plasma TM levels were significantly higher for dogs with leishmaniasis, especially those with severe clinical signs (Group II). In this study a significant relationship between high plasma TM levels and beta and gamma globulins plasma levels was observed. These elevated concentrations of gamma-globulins can be explained as a polyclonal response of B lymphocytes. These antibodies are not all directed against Leishmania but also reflect an autoimmune reaction. Deposition of circulating immuno-complexes (type III hypersensitivity) in blood vessels of different organs such

TABLE I
Hematological and biochemical findings observed in healthy dogs and three groups of sick dogs

	Healthy dogs	Group I	Group II
Wbc (10^3/mm^3)	10.2 ± 4.5	9.6 ± 3.2	8.14 ± 2.9
Rbc (10^6/mm^3)	5.8 ± 0.4	6.7 ± 0.4	4.86 ± 1.6
Hgb (g%)	13.5 ± 1.0	16.3 ± 1.3	10.9 ± 3.5
Hct (%)	38.6 ± 2.8	44.5 ± 2.6	31.5 ± 11.2
MCV (μ^3)	67.2 ± 3.3	66.1 ± 2.1	64.3 ± 3.7
MCHC ($\mu\mu$g)	24.1 ± 1.4	24.2 ± 1.1	23.5 ± 1.5
MCH (%)	35.8 ± 2.9	36.6 ± 2.2	36.5 ± 2.4
Plt (10^3/mm^3)	273.2 ± 85.3	224.9 ± 74.6	189.2 ± 103.3
TM (ng/ml)	2.51 ± 0.5	2.9 ± 0.4	3.63 ± 0.9*
PT (sec)	7.4 ± 1.1	8.0 ± 2.3	8.2 ± 1.8
aPTT (sec)	12.6 ± 4.1	13.1 ± 3.7	16.6 ± 3
Fibr. (mg/dl)	301.7 ± 42.8	235.6 ± 39.8	282.7 ± 104.7
Urea (mg/dl)	30 ± 15	40.3 ± 9.4	40 ± 9.1
Creatinine (mg/dl)	0.6 ± 0.1	1.1 ± 0.3	1.3 ± 0.4
ALT (UI/L)	36 ± 11	44.2 ± 13.4	69.15 ± 25
Prot. T. (g/dl)	6.0 ± 0.5	7.0 ± 0.8	7.99 ± 1.3
Alb. (g/dl)	3.5 ± 0.2	3.3 ± 0.5	2.5 ± 0.6
Alfa 1 (g/dl)	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Alfa 2 (g/dl)	0.5 ± 0.1	0.7 ± 0.2	0.7 ± 0.2
Beta 1 (g/dl)	0.4 ± 0.2	0.9 ± 0.3	1.06 ± 0.2
Beta 2 (g/dl)	0.6 ± 0.2	0.9 ± 0.4	1.25 ± 0.5
Gamma (g/dl)	0.5 ± 0.3	0.8 ± 0.2	2.19 ± 1.5

* $p \leq 0.01$.

as those of the liver, spleen, skin, kidney, etc cause diffuse microvasculites and consequently, damage to the endothelium. The skin, ocular and haemopoietic involvement observed for animals of the second group is suggestive of an immunomediated process of the vessels with endothelial damage and TM increase. In agreement with Kumada *et al.* (1988), the increase of ALT in the second group suggests a decrease in hepatic clearance and, consequently, an increase in plasma TM levels. In human internal medicine, high levels of TM have also been reported for the thromboembolic condition and for renal failure which have also been reported for canine leishmaniasis (Font *et al.*, 1993; Ciaramella *et al.*, 1997). However, in our research, the basal levels of PT, APTT, fibrinogen, urea and creatinine excluded renal failure and/or hemocoagulopathy. In conclusion, despite the low number of dogs used, our results suggest that plasma thrombomodulin can also be used as a non-invasive marker for endothelial injury in dogs as well as in humans. Further studies, including immunohistochemistry and the value of other endothelium markers, are needed to assess the role

played by TM in the genesis of endothelial lesions in dogs suffering from physiological and pathological conditions.

REFERENCES

Ciaramella, P., Oliva, G., De Luna, R., Gradoni, L., Ambrosio, R., Cortese, L., Scalone, A. and Persechino A., 1997. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*. *Veterinary Record*, **141**, 539–542

Font, A., Closa, J.M., Molina, A. and Mascort, J., 1993. Thrombosis and nephrotic syndrome in a dog with a visceral leishmaniasis. *Journal of Small Animal Practice*, **34**, 466–470

Kumada, T., Dittman, W.A. and Majerus, P.W., 1988. A role of thrombomodulin in the pathogenesis of thrombin-induced thromboembolism in mice. *Blood*, **71**, 728–733

Pumarola, M., Brevik, L., Badiola, S., Vargas, A., Domingo, M. and Ferrer, L., 1991. *Journal Comparative Pathology*, **105**, 209–214.

Veterinary Research Communications, 28 (2004) 331–334

Haemostatic Disorders in Dogs Naturally Infected by *Leishmania infantum*

[1]M. Corona[1], P. Ciaramella[1]*, A. Pelagalli[2], L. Cortese[1], M.E. Pero[2], D. Santoro[1] and P. Lombardi[2]
[1]*Department of Veterinary Clinical Science – Section of Internal Medicine;*
[2]*Department of Biological Structure, Function and Technologies – University of Naples Federico II, via Delpino, 1-80137 Naples, Italy*
* *Correspondence: Dipartimento di Scienze Cliniche Veterinarie – Sezione di Clinica Medica – Facoltà di Medicina Veterinaria, via Delpino, 1-80137 Napoli, Italy*
E-mail: paociara@unina.it

Keywords: Leishmaniasis, platelet, aggregation, dog

Abbreviations: CanL, canine Leishmaniasis; TT, prolonged thrombin time; APTT, activated partial thromboplastin time; IFAT, immunofluorescent antibody test; TP, total plasma proteins; ALT, alanine transaminase; PT, prothrombin time

INTRODUCTION

Haemostatic abnormalities such as epistaxis, haematuria and haemorrhagic diarrhoea, have been reported in canine Leishmaniasis (CanL) (Ciaramella *et al*., 1997). Thrombocytopenia, thrombocytopathy, prolonged thrombin time (TT), prolonged activated partial thromboplastin time (APTT) and increased fibrinogen/fibrin degradation products have been found on *Leishmania* infection, and indicate that this parasite affects primary haemostasis, coagulation, and fibrinolysis (Moreno, 1999). The effect on haemostasis is complex and not all the haemostasis alterations reported in the literature can be found at once in a dog with leishmaniasis because of the typical clinical polymorphism of the disease. The pathogenesis of these disorders is not well known but, in human leishmaniasis, it has been attributed to necrotising vasculitis, renal and/or hepatic failure and immune-mediated disorders. The object of this study was to evaluate platelet function and secondary haemostasis in dogs naturally infected by *Leishmania infantum* with different grades of clinical involvement, in order to improve knowledge of the haemostatic disorders found for this protozoan disease.

MATERIALS AND METHODS

30 dogs naturally infected by *Leishmania infantum* and a control group of 10 healthy dogs were examined. None of the infected dogs had ever undergone any specific anti-Leishmania therapy. Sick animals were divided into three groups of 10 dogs on the

basis of the clinical examination (Ciaramella *et al*., 1997): first group – oligo-symptomatic dogs; second group – symptomatic dogs; third group – markedly symptomatic dogs. The clinical diagnosis was always confirmed by direct observation of the protozoan in Giemsa stained fine needle aspirates of bone marrow and lymph nodes, and serologically using the immunofluorescent antibody test (IFAT). The threshold titre for positivity was 1/160. A full blood count and measurement of total plasma proteins (TP), serum protein electrophoresis, alanine transaminase (ALT), creatinine and urea serum levels were obtained for each dog. Prothrombin time (PT) and activated partial thromboplastin time were determined twice using a semi-automatic coagulometer. The aggregation response of platelet-rich plasma was measured using a turbid metric method with a Chronolog aggregometer after addition of collagen type I calf skin and adenosine 5′-diphosphate (ADP) suspension. The collagen and ADP suspension were used individually at doses within a range of 2.5–200 μg/ml and 2.5–10 μM. Mean values with their standard deviations were calculated from the data obtained and then statistically evaluated using one-way analysis of variance. Significant results were subjected to the Student–Newman–Keuls Multiple Comparisons Test.

RESULTS

Compared with the control group, the maximum relative aggregation response decreased significantly in each of the groups at the various doses employed. Decrease in platelet aggregation using collagen was more evident than that observed with ADP agonist (ADP-vs-collagen 83.7 vs 78.4%). When higher doses of collagen were employed the third group showed a significant decrease in aggregation response to collagen as compared to the first and second groups (Table I). A reduction in haemoglobin plasma levels and an increase in urea and creatinine plasma levels were observed for the third group; the same group showed moderate thrombocytopenia, while no difference in platelet count was found in the other groups of sick dogs (Table I). A longer APTT was observed for the second and third groups as compared with the control and the first groups. Finally, the A/G ratio dropped progressively in all three groups of infected dogs.

DISCUSSION

As previously reported under natural and experimental conditions (Moreno *et al*., 1998; Valladares *et al*., 1998), our results also show that a significant reduction in platelet aggregation can be found in CanL.

In agreement with previous preliminary work (Ciaramella *et al*., 2002), the reduction in platelet aggregation produced by the two agonists was different. The greater sensitivity of collagen as compared with ADP can be explained by considering their

TABLE I
Haematological and biochemical findings observed in for healthy dogs and three groups of sick dogs

	Healthy dogs (n. 10)	Group I (n. 10)	Group II (n. 10)	Group III (n. 10)
Plt (10^3/μl)	273.2 ± 85.3^{a}	225 ± 82.4	228 ± 96.3	145 ± 60^{b}
ADP[1] (%)	93.4	83.16	84.17	83.78
Collagen[1] (%)	94.3	82.19	77.91	73.23
PT (sec.)	7.4 ± 1.1	8 ± 1.9	7.6 ± 0.8	8.3 ± 1.9
APTT (sec.)	13.7 ± 1.0^{A}	13.4 ± 3.0^{A}	16.1 ± 4.5	18.6 ± 1.2^{B}
Urea (mg/dl)	32 ± 9.1^{B}	40.7 ± 9.6^{B}	36.4 ± 10.2^{B}	67.4 ± 13.3^{A}
Creatinine (mg/dl)	0.8 ± 0.2^{B}	0.9 ± 0.3^{B}	1.1 ± 0.2^{B}	1.9 ± 0.6^{A}
ALT (UI/L)	31 ± 5.8^{B}	37 ± 16.9^{B}	45.7 ± 17^{B}	80.4 ± 16.3^{A}
T.P. (g/dl)	6.2 ± 0.2^{A}	6.8 ± 0.6^{a}	8.1 ± 1.4^{Bb}	7.9 ± 1.1^{Bb}
Albumin (g/dl)	3.1 ± 0.5^{b}	3.2 ± 0.5^{b}	2.8 ± 0.5	2.4 ± 0.6^{a}
Alfa 1 (g/dl)	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Alfa 2 (g/dl)	0.3 ± 0.1^{A}	0.7 ± 0.2^{BCb}	0.6 ± 0.1^{B}	0.9 ± 0.1^{Ca}
Beta 1 (g/dl)	0.6 ± 0.1^{A}	0.8 ± 0.2	1.0 ± 0.3^{B}	1.0 ± 0.2^{B}
Beta 2 (g/dl)	0.5 ± 0.2^{Aa}	0.9 ± 0.3^{b}	1.2 ± 0.5^{B}	1.3 ± 0.4^{B}
Gamma (g/dl)	0.8 ± 0.2^{B}	1.0 ± 0.3^{B}	2.2 ± 0.9^{A}	2.1 ± 1.0^{A}
A/G	1	0.80	0.53	0.44

A,B,C, Means within a line with the same letter are not significantly different ($p \leq 0.01$).
a,b,c, Means within a line with the same letter are not significantly different ($p \leq 0.05$).
[1] Values indicated refer to the ADP and Collagen maximun doses employed.

different affinities for the membrane receptors: ADP agonist interacts with the GPIIb/IIIa membrane receptor, while collagen fastens especially to the GPIa/IIa membrane receptor. It is possible that the parasite causes direct damage to the different platelet membrane receptors which cannot then be recognised as "self" and consequently activate the immune-mediated process. Recently Dominguez and Torano (2001) have demonstrated that in the first phases of infection *Leishmania* is able to interact directly with the platelets by a specific mechanism termed "immune adherence", with the formation of large aggregates. Our data suggest that in dogs with severe clinical symptoms there is involvement of the intrinsic pathway as demonstrated by the prolonged APTT levels. This involvement could be due to a reduction in synthesis together with an increase in consumption of one or more clotting factors following hepatic damage and the chronic inflammatory state. Similar results were reported by Moreno (1999) for 26 dogs with Leishmaniasis although this author has not related his results to clinical conditions. In conclusion, this study demonstrates that in natural CanL primary and secondary haemostatic alterations are related to the severity of clinical symptoms. Collagen agonist could be used as a non-invasive first choice marker for the evaluation of platelet aggregation in a *Leishmania* infection.

In animals with very severe clinical conditions, the compromised immune state, the chronic inflammatory condition and the progressive liver and renal damage influence platelet function and the synthesis and metabolism of the clotting factors and can cause future bleeding disorders.

REFERENCES

Ciaramella, P., Oliva, G., De Luna, R., Gradoni, L., Ambrosio, R., Cortese, L., Scalone, A. and Persechino, A., 1997. A retrospective clinical study of canine leishmaniasis in dogs naturally infected by *Leishmania infantum*. *Veterinary Record*, **141**, 539–542

Ciaramella, P., Pelagalli, A., Cortese, L., Pero, M.E., Corona, M., Lombardi, P. and Avallone, L., 2002. Platelet aggregation response in canine leishmaniasis. *Platelets*, **13**, 331

Dominguez, M. and Torano, A., 2001. Leishmania immune adherence reaction in vertebrates. *Parasite Immunology*, **23**, 259–265

Moreno, P., 1999. Evaluation of secondary haemostasis in canine leishmaniasis. *Veterinary Record*, **144**, 169–171

Moreno, P., Lucena, R. and Ginel, J., 1998. Evaluation of primary haemostasis in canine leishmaniasis. *Veterinary Record*, **142**, 81–83

Valladares, J.E., Gopegui, R.R., Riera, C., Alberola, J., Gallego, M., Portus, M. and Arboix, M., 1998. Study of haemostatic disorders in experimentally induced leishmaniasis in Beagle dogs. *Research in Veterinary Science*, **64**, 195–198

Veterinary Research Communications, 28 (2004) 335–338

MPA Inhibits Idarubicin Activity on Cu-Zn SOD and Catalase

L. Crispino[1], R. Ciarcia[1]*, S. Montagnaro[2], R. Pagnini[1], F. Pacelli[2] and S. Florio[1]
[1] *Dipartimento di Strutture, Funzioni e Tecnologie Biologiche e;* [2] *Dipartimento di Patologia e Sanità Animale – Università di Napoli "Federico II", Via Veterinaria, 1-80137 Napoli, Italy*
* *Correspondence: Dipartimento di Strutture, Funzioni e Tecnologie Biologiche, Università di Napoli "Federico II", Via Veterinaria, 1-80137 Napoli, Italy*
E-mail:robertociarcia@virgilio.it

Keywords: catalase, Cu- Zn SOD, rat

Abbreviations: IDA, idarubicin; CACS, cancer-related anorexia-cachexia syndrome; MPA, medroxyprogesterone acetate; MA, megestrol acetate; IL-I, interleukin-I; IL-6, interleukin 6; MDA, malondialdehyde; CAT, catalase; Cu-Zn SOD, copper-zinc superoxide dismutase

INTRODUCTION

It is known that serum levels of cytokine, including interleukin-I (IL-I) and interleukin 6 (IL-6) are very high in cancer patients and are related to oxidative stress. These cytokines play a key role in the pathogenesis of cancer-related anorexia-cachexia syndrome (CACS) induced both by chronic administration of chemotherapeutic drugs and the tumour itself. Recently, Mantovani *et al.* (1998) demonstrated that high doses of Medroxyprogesterone acetate (MPA), a semisynthetic progestin, and Megestrol acetate (MA) reduce serum levels of interleukin-I (IL-I) and interleukin 6 (IL-6) *in vivo* for cancer patients with CACS leading to beneficial therapeutic effects. Moreover, clinical use of anthracyclines for the treatment of several malignant tumours has been limited both by a dose-dependent cardiotoxicity and drug resistance. Therefore, the clinical use of such chemosensitizers remains questionable and the identification of more active but less toxic compounds is needed. Our recent studies, in chronic lymphatic leukaemia cells, have demonstrated that MPA reduces Malondialdehyde (MDA) levels increased by anthracyclines (Pagnini *et al*., 2000). In this study we evaluated the activity of two myocardial antioxidant enzymes, copper-zinc superoxide dismutase (Cu-Zn SOD) and catalase (CAT) in the plasma of rats treated *per os* for 15 days with Idarubicin (IDA) and Medroxyprogesterone acetate (MPA), either used alone or in combination.

MATERIALS AND METHODS

Idarubicin (IDA) and Medroxyprogesterone acetate (MPA) were obtained from Pharmacia-Upjohn (Milan, Italy), Phenazine methosulphate (MPS), Nitro blue tetrazolium (NBT), EDTA, NADH, Cu-Zn SOD, Catalase, hydrogen peroxide (H_2O_2) and Potassium dichromate ($K_2Cr_2O_7$) were obtained from SIGMA (Milan, Italy).

120 stalling male Wistar rats (Harlan-Nossan – Correzzana, Milan, Italy) weighing between 110–130 g, were subdivided into four groups and treated orally each day for 15 days as follows:

Group I: water in 5 ml/kg;
Group II: IDA 0.25 mg/kg;
Group III: MPA 25 mg/kg;
Group IV: IDA 0.25 mg/kg + MPA 25 mg/kg.

Two hours after the last treatment, rats were killed by decapitation, blood was collected in heparinized tubes and was centrifuged at 13,000 *g* for 3 min.

Cu-Zn SOD activity

The spectrophotometric assay, as described by Ewing and Janero (1995), was applied to evaluate Cu-Zn SOD activity. The assay is based on spectrophotometric assessment of O_2^- mediated nitro blue tetrazolium reduction by an aerobic mixture of NADH and phenazine methosulphate, which produces superoxide chemically at non-acidic pH. Absorbance was read at 520 nm using a Perkin Elmer UV Spectrophotometer.

Catalase activity

The colorimetric assay, described by Sinha (1971) (4) was applied to evaluate Catalase activity. This method is based on the fact that dichromate is reduced to chromate acetate when heated in acetic acid in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically and absorbance is read at 570 nm.

RESULTS

Table I shows the effects of IDA (0.25 mg/kg/day) and MPA (25 mg/kg/day) used alone or in combination, on Catalase activity. The calalase decreased by $42.7 \pm 8.4\%$ in plasma of rats treated with IDA but MPA alone did not modify this parameter. MPA used in combination with IDA reduced the inhibition activity of anthracyclines on Catalase. The inhibition shifted from $42.7 \pm 8.4\%$ (IDA alone) to $25.5 \pm 9.47\%$ (IDA + MPA).The effects of IDA (0.25 mg/kg/day) and MPA (25 mg/kg/day) used alone or in combination, on Cu-Zn SOD activity are shown in Table II. MPA reduced the inhibition activity of the anthracyclines on Cu-Zn SOD. The inhibition shifted from $31.5 \pm 4.41\%$ (IDA alone) to $20.5 \pm 3.34\%$ (IDA + MPA).

TABLE I
Catalase activity in plasma of rats treated or not treated *per os* for 15 days with IDA 0.25 mg/kg/day, MPA 25 mg/kg/day, or with IDA 0.25 mg/kg/day + MPA 25 mg/kg/day. Data are expressed as D.O. obtained after 10 min of incubation. Data express the IDA values (means ± SD) obtained from five experiments performed in duplicate. *$p < 0.05$ vs. basal levels; **$p < 0.05$ vs IDA treatment

Group	Treatment	D.O.	Δ %	p*	p**
I	Control	1.05 ± 0.213	–	–	–
II	IDA	0.61 ± 0.314	-42.7 ± 8.4	<0.05	–
III	MPA	1.19 ± 0.239	$+3.84 \pm 0.77$	–	–
IV	IDA + MPA	0.78 ± 0.291	-25.5 ± 9.47	<0.05	<0.05

TABLE II
Cu-Zn SOD activity in plasma of rats treated or not treated *per os* for 15 days with IDA 0.25 mg/kg/day, MPA 25 mg/kg/day, or with IDA 0.25 mg/kg/day + MPA 25 mg/kg/day. Data are expressed as D.O. obtained after 5 min of incubation. Data show the IDA values (means ± SD) obtained from five experiments performed in duplicate. *$p < 0.05$ vs. basal levels; **$p < 0.05$ vs IDA treatment

Group	Treatment	D.O.	Δ %	p*	p**
I	Control	0.17 ± 0.031	–	–	–
II	IDA	0.12 ± 0.025	-31.5 ± 4.47	<0.05	–
III	MPA	0.18 ± 0.029	$+2.9 \pm 0.61$	–	–
IV	IDA + MPA	0.14 ± 0.023	-20.5 ± 3.34	<0.05	<0.05

DISCUSSION

Clinical and experimental data suggest that high concentrations of reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) can cause cellular damage and may play a key role in drug-induced toxicity (Lesko *et al*., 1980). Such oxidative stress is not only responsible for the cancer genesis but also for both an anthracycline-induced toxicity effect and anorexia/cachexia. It has been reported that, as the cancer-related anorexia/cachexia syndrome (CACS) is correlated with cytokine levels, it is consequently also correlated with oxidative stress. It has been recently demonstrated that high doses of Medroxyprogesterone acetate (MPA) and Megestrol acetate provide protection from CACS by modulating cytokines and serotonin production and that such an effect is related to oxidative stress. Our results showed that high doses of MPA reduce the inhibition of Cu-Zn SOD and Catalase induced by idarubicin. In conclusion, these data are in agreement with those of other authors, showing that IDA-induced oxidative stress can be reduced by administration

of high doses of MPA. In this way, both reduction in cardiotoxicity and beneficial therapeutic effects can be achieved.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. Manco Leonida for his excellent technical assistance.

REFERENCES

Ewing, J.F. and Janero, D.R., 1995. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Analytical Biochemistry*, **232**, 243–248

Lesko, S.A., Lorentzen, R.J. and Ts'o, P.O., 1980. Role of superoxide in deoxyribonucleic acid strand scission. *Biochemistry*, **19**, 3023–3028

Mantovani, G., Maccio, A., Lai, P., Massa, E., Ghiani, M. and Santona, M.C., 1998. Cytokine activity in cancer-related anorexia/cachexia: role of megestrol acetate and medroxyprogesterone acetate. *Seminar Oncology*, **25**, 45–52

Pagnini, U., Pacilio, C., Florio, S., Crispino, A., Claudio, P.P., Giordano, A. and Pagnini, G., 2000. Medroxyprogesterone acetate increases anthracyclines uptake in chronic lymphatic leukemia cells: role of nitric oxide and lipid peroxidation. *Anticancer Research*, **20**, 33–42

Sinha, A.K., 1972. Colorimetric assay of catalase. *Analytical Biochemistry*, **47**, 389–394

Veterinary Research Communications, 28 (2004) 339–342

Breed Distribution of Canine Diabetes Mellitus in Italy

F. Fracassi[1], M. Pietra[1], A. Boari[2], G. Aste[2], M. Giunti[1] and P. Famigli-Bergamini[1]*
[1] *Department of Veterinary Clinical Sciences, Internal Medicine Section, Alma Mater Studiorum-University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano dell'Emilia (Bo), Italy;* [2] *Department of Veterinary Clinical Sciences, Internal Medicine Section, University of Teramo, V.le Crispi 212, 64020 Teramo, Italy;*
* *Correspondence: Dipartimento Clinico Veterinario, Alma Mater Studiorum-University of Bologna, via Tolara di Sopra 50, 40064, Ozzano dell'Emilia (Bo), Italy*
E-mail:FAMIGLI@vet.unibo.it

Keywords: dog, diabetes mellitus, epidemiology, breed, statistical analysis

INTRODUCTION

In human medicine the genetic components of insulin-dependent diabetes mellitus (IDDM) have been investigated, and it is well known that many genes are involved in the pathogenesis of IDDM (Davies *et al*., 1994). These genes, mainly belonging to the major histocompatibility complex (MHC), seem to enhance the development of antipancreatic T lymphocytes (Zamani *et al*., 1998). Although there is evidence for the inheritance and immune mediated pathogenesis of IDDM in dogs, the genes involved in the spontaneous disease have not been identified and may vary from breed to breed (Gershwin, 1975; Kramer *et al*., 1988; Elie and Hoenig, 1995; Klimmer *et al*., 2002). A familial predisposition for IDDM has been described for Samoyeds (Kimmler *et al*., 2002), Keeshonds (Kramer *et al*., 1988), Golden Retrievers (Williams *et al*., 1980) and Poodles (Gershwin, 1975). Epidemiological studies on the risk of developing diabetes mellitus (DM) in dogs have mainly been focused on North American canine populations (Marmor *et al*., 1982; Hess *et al*., 2000). To the best of our knowledge only one epidemiological survey has been carried out in Europe (Doxey *et al*., 1985). Unfortunately this study did not show the results obtained in detail and probably for this reason very common Italian breeds were not mentioned. From anecdotal reports, some breeds such as the English Setter and Irish Setter may be at high risk of developing DM in Italy.

The aim of this study was to evaluate the breed distribution and the risk of developing DM in an Italian canine population. The identification of these breeds may further direct studies concerning pedigree analyses, genetic aspects and mode of inheritance of this disease.

MATERIALS AND METHODS

By running a computer search of all dogs admitted to the Department of Veterinary Clinical Sciences of the Alma Mater Studiorum-University of Bologna between

January 1995 and December 2002, it was possible to identify 225 dogs diagnosed with DM. Medical records regarding the physical examination, urinalysis and biochemistry were reviewed in detail for each dog and 186 dogs with DM were used for the study. Inclusion criteria consisted of persistent hyperglycemia with glycosuria and at least two common clinical signs of DM (polyuria, polydipsia, weight loss or polyphagia) or glucosuria with ketonuria. 39 dogs were excluded from the study because they had insufficient data for diagnosis (a single result of either hyperglycemia or glycosuria without clinical information about the dog), or transient (diestral) or secondary DM. At the end of this selection, the case series included 186 dogs with DM that had been admitted to our Teaching Hospital between January 1995 and December 2002. 13857 dogs without DM had been admitted to the Department of Veterinary Clinical Sciences during the same period and this was considered the comparison group. Of 135 different breeds (including the mixed-breed category) admitted to the Clinic over the period of the study, 23 (including the mixed-breed category) had at least one dog with DM. Statistical analysis was restricted to those breeds where the sum of observed and expected values for diabetic subjects (calculated by multiplying the number of subjects in each breed by the percentage of diabetics in the whole population studied) was > 4. This procedure ensured that a sufficient numbers of dogs was considered in order to document breeds at low and high risk of developing DM. The odds ratio was calculated for each breed by choosing mixed breed dogs as the reference breed (odds ratio $= 1$). A 95% confidence interval was also calculated. A value of $p < 0.05$ was considered significant and < 0.001 highly significant.

RESULTS AND DISCUSSION

13 breeds showed a sum of observed and expected values > 4. The following 11 breeds of diabetic dogs did not meet the criteria and were consequently excluded from the statistical analysis: Samoyeds (2 dogs), Lagotto (2), Maltese (2), Pug (1), Collie (1), Corsican (1), Fox Terrier (1), Miniature Schnauzer (1), Shih Tzu (1), Newfoundland (1), Pomeranian (1). The Boxer breed was included because it was highly represented (666) and also showed an expected count > 4 (8.82), despite the fact that no diabetic dogs were present (observed count $= 0$). In our survey, the Irish Setter was the breed with the highest risk of developing DM, having an odds ratio 3.91 times greater than that of the control group (mixed breed dogs). The following breeds were also found to be at high risk of developing DM: Poodle (O.R. $= 2.80$; $p < 0.001$), Yorkshire Terrier (O.R. $= 2.62$; $p < 0.001$) and English Setter (O.R. $= 2.60$; $p < 0.001$). The breeds with the lowest predisposition to the disease (DM) were: Boxer (O.R. $= 0$; $p < 0.05$), German Shepherd (O.R. $= 0.11$; $p < 0.001$) and Doberman Pinscher (O.R. $= 0.13$; $p < 0.05$).

Among the breeds that had sufficient numbers of dogs to meet our sample size criteria for analysis, our results showed that four dog breeds are at high risk and three dog breeds are at low risk for developing DM. While our data for the Yorkshire

Terrier and Poodle are substantially in agreement with the results obtained by other authors (Doxey *et al*., 1985; Hess *et al*., 2000), our study also suggested that Irish and English Setters are respectively 3.9 and 2.6 times more likely to develop DM than mixed breed dogs. The low risk documented for German Shepherd, Boxer and Doberman Pinscher is in agreement with previous reports (Hess *et al*., 2000; Marmor *et al*., 1982).

The reason for the differences between the results of our study and those of other previously published reports, could be due either to the different distribution/representation of canine breeds in different geographic locations or to the presence of different genetic components of IDDM within the same breed (Gershwin, 1975).

A further limitation of our survey may be that the study population (percentage of diabetic subjects over the entire population examined = 1.33%) may not accurately reflect the population of diabetic dogs at large. This may be because the Department of Veterinary Clinical Sciences of the University of Bologna is considered as a referral centre for DM. To the best of our knowledge, this is the first large European epidemiological study conducted for evaluation of the risk of DM development in canine breeds spread over the Italian territory. In conclusion our study suggests that Irish Setter and English Setter dogs should be added to the list of breeds at high risk of developing DM, at least while considering the Italian situation. In our opinion, these results constitute an important starting point for further research regarding pedigree analyses, genetic aspects and mode of inheritance of DM in the dog.

REFERENCIES

Davies, J.L., Kawaguchi, Y., Bennet, S.T., Copeman, J.B., Cordell, H.G., Pritchard, L.E., Reed, P.W., Gough, S.C.L., Jenkins, S.C, Palmer, S.M., Balfour, K.M., Rowe, B.R., Farrall, M., Barnett, A.H., Bain, S.C. and Todd, J.A., 1984. A genome-wide search for human type 1 diebetes susceptibility genes. *Nature*, **371**, 130–136

Doxey, D.L., Milne, E.M. and Mackenzie, C.P., 1985. Canine diabetes mellitus: a retrospective survey. *Journal of Small Animal Practice*, **26**, 555–561

Elie, M. and Hoenig, M., 1995. Canine immune-mediated diabetes mellitus: a case report. *Journal of the American Animal Hospital Association*, **31**, 295–299

Gershwin, L.J., 1975. Familiar canine diabetes mellitus. *Journal of American Veterinary Medical Association*, **167**, 479–480

Hess, R.S., Kass, P.K. and Wart, C.R., 2000. Breed distribution of dogs with diabetes mellitus admitted to a tertiary care facility. *Journal of American Veterinary Medical Association*, **9**, 1414–1417

Hoenig, M. and Dawe, D.L., 1992. A qualitative assay for beta cell antibodies. Preliminary results in dogs with diabetes mellitus. *Veterinary Immunology and Immunopathology*, **32**, 195–203

Kimmler, S.E., Ward, C.R., Henthorn, P.S. and Hess, R.S., 2002. Familial insulin-dependent diabetes mellitus in Samoyed dogs. *Journal of the American Animal Hospital Association*, **38**, 235–238

Kramer, J.W., Klaassen, J.K., Baskin, D.G., Prieur, D.J., Rantanen, N.W., Rubinette, J.D. and Graber, W.R., 1988. Inheritance of diabetes mellitus in Keeshond dogs. *American Journal of Veterinary Research*, **49**, 428–431

Marmor, M., Willeberg, P., Glickman, L.T., Priester, W.A., Cypess, R.H. and Hurvitz, A.I., 1982. Epizootiologic patterns of diabetes mellitus in dogs. *American Journal of Veterinary Research*, **43**, 465–470

Williams, M., Gregory, R., Schall, W, Rovner, D. and Padgett, G., 1980. Diabetes mellitus in a colony of golden retrievers. *Federation-Proceedings*, **39**, 637

Zamani, M. and Cassiman, J.J., 1998. Reevaluation of the importance of polymorphic HLA class II alleles and amino acids in the susceptibility of individuals of different populations to type I diabetes. *American Journal of Medical Genetics*, **76**, 183–194

Veterinary Research Communications, 28 (2004) 343–346

Clinic and Ultrasonographic Findings in a Cat with Tetralogy of Fallot

A. Fruganti*, M. Cerquetella, F. Beribè, A. Spaterna and B. Tesei
Veterinary Science Department – Clinical Section – Faculty of Veterinary Medicine, University of Camerino, 62024 Matelica, Macerata, Marche, Italy
**Correspondence: Dipartimento di Scienze Veterinarie – Sezione clinica – Università degli studi Camerino, 62024 Matelica, (MC), Marche, Italy*
E-mail: alessandro.fruganti@unicam.it

Keywords: cat, tetralogy of Fallot, echocardiography, Doppler

Abbreviations: TOF, Tetralogy of Fallot

INTRODUCTION

Tetralogy of Fallot (TOF) is a congenital cardiopathy of man and domestic animals. Fallot was a cardiologist in human medicine of the late 19th–early 20th century. This cardiac malformation is characterized by ventricular septal defect, dextroposition of the aorta, pulmonic stenosis and consequent right ventricular hypertrophy. Cardiac morphological alterations in TOF derive from a defective fetal development of both pulmonary and aortic arteries, resulting from *troncus arteriosus*, and right and left ventricular outflow tracts, resulting from *conus arteriosus*. There is also an incomplete development of the conotruncal septum which causes a skew between dorsal and apical parts of interventricular septum.

As shown by Goodwin and Cooper (1992), pulmonary stenosis causes an increased resistance to blood flow ejected from the right ventricle. This leads to hypertrophy and hypertension of this ventricle, and leads to a right-to-left shunt through interventricular septum defect. The first consequence of the shunt is an inflow in aorta of bad oxygenated blood, that can easily induce hypoxia. Symptoms are failure to grow, exercise intolerance, cyanosis and syncope. Cyanosis depends on the entity of the shunt and it is not always present, but it is typical of most cases. For this reason, in the past the illness was called "blue disease" because of the colour assumed by mucosae. Cyanosis is always present when the subject gets excited or during physical effort, when even asphyxia can occur.

TOF is more common for dogs than for cats (Patterson *et al*., 1993). For this species diagnosis is performed by anatomo-pathological examination or angiography which is an invasive *intra vitam* examination involving a lot of risks (Eyster *et al*., 1977). The aim of this report is to furnish a contribution to the study of this pathology in cats, especially for the non-invasive diagnostic methods such as ultrasound.

MATERIALS AND METHODS

The subject of the study is a three-year old male European cat. Anamnesis was of depression, hypokinesia, increased respiratory effort, even with light physical activity, and lipothymic crises characterized by loss of standing position and unconsciousness. The lipothymic crises and dyspnea were related to the intensity of physical activity. The cat underwent physical, electrocardiographic, radiographic and ultrasonographic examination. It was impossible to collect a blood sample because of the occurrence of respiratory crisis during sampling. Electrocardiography was performed with the cat in right lateral recumbency, using a Nihon Kohden – Eclaps 12 – ECG 8110R instrument. Radiography was performed with a latero-lateral projection (left to right). M-mode, b-mode and Doppler echocardiographic evaluations were performed through the clipped right parasternal window and clipped apical and caudal left parasternal windows. The ultrasound machines used were a Kontron "Vetson Color", with a micro-convex 6.5 MHz probe and a Medison AS-8800, provided with a micro-convex multi-frequency (8–5 MHz) transducer. Three months after the first visit the subject was visited and examined again. This time it was possible to take a blood sample with anticoagulant which was processed using an automatic cell counter (Abbot "Cell-Dyn 3500") to obtain the hemogram.

RESULTS

During the first observation, clinical examination led us to identify dyspnea and cyanosis which was particularly evident on the nose, ears and mucosas. The intensity of the dyspnea and cyanosis increased as soon as the subject became more excited because of the inevitable handling during the visit. It was possible to identify a weak and frequent pulse (240/1), and a diffuse thrill, synchronous with the cardiac apex beat. A systolic harsh murmur, of grade 4–5/6, best heard in the pulmonic valve area and also on the right side of the chest (third intercostal space) was also evident. Clinical findings were almost the same during the second visit, although the systolic murmur was less loud (grade 3–4/6). The electrocardiogram showed a right ventricular enlargement and the radiography showed a slight reduction of the left atrium volume. Mono- and bi-dimensional echocardiography showed a large ventricular septal defect (Fig. 1), a dextroposition of the aorta ("overriding aorta") (Fig. 2) producing stenosis of the right ventricle outflow tract (pulmonic stenosis) and, lastly, a hypertrophy of the interventricular septum and of the right ventricle wall. Color Doppler examination showed a turbulent blood flow in the pulmonary artery and through the ventricular septal defect with a right-to-left shunt. Hemogram results showed polycythemia (HCT = 70.4%, RBC = $16.8 \times 106/mm^3$, HGB = 23.6 g/dL) as compared to the normal limits (Feldman *et al.*, 2000).

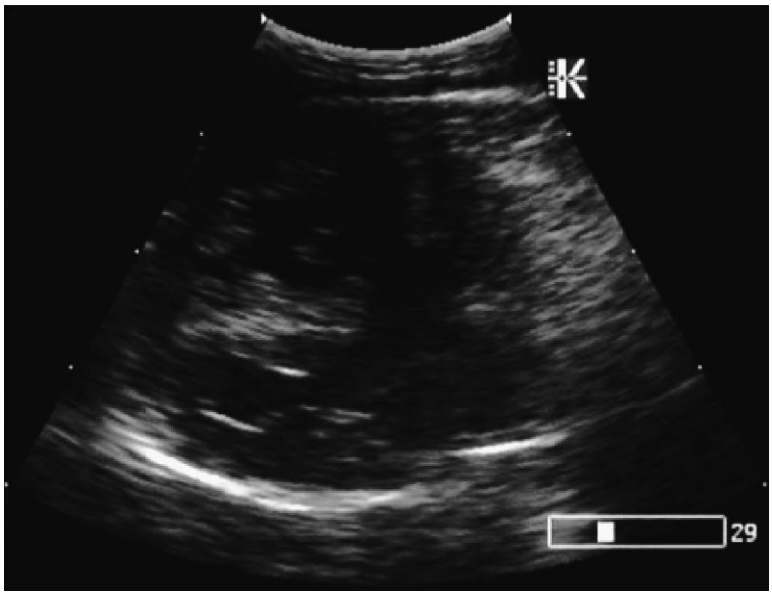


Figure 1. Right parasternal, long-axis 4 chamber view: large ventricular septal defect

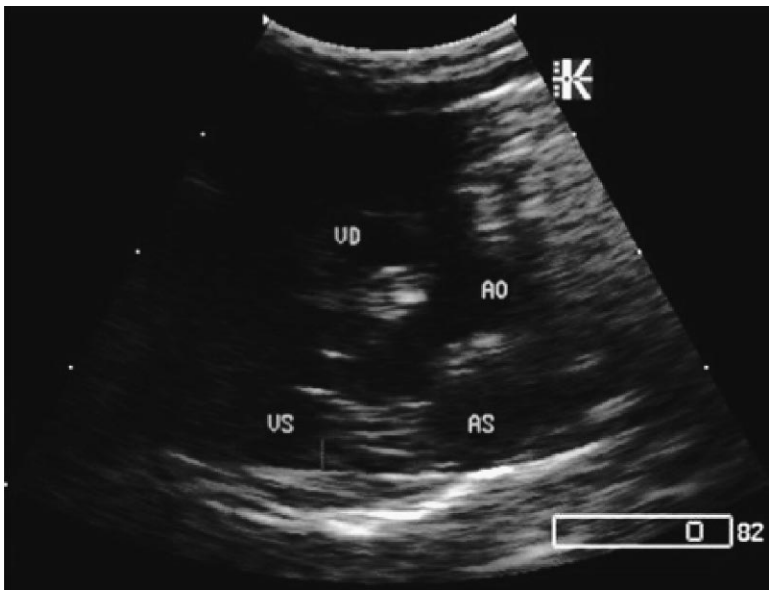


Figure 1. Right parasternal, long-axis LV outflow view: “overriding aorta”

DISCUSSION

The congenital cardiopathy suspected during physical examination was confirmed by instrumental and laboratory tests. The test that appeared to be most useful for confirmation of the diagnosis of tetralogy of Fallot was echocardiography. In fact ultrasonography gave a correct intra vitam diagnosis, despite the fact that the probes normally used in veterinary medicine are not very adaptable to small acoustic windows. The fact that the murmur was also heard on the right side of the chest is probably due to the radiation of the murmur caused by pulmonic stenosis and/or to the blood flow through the ventricular septal defect or through the ascending tract of the "overriding aorta". This kind of murmur is less loud when the ventricular septal defect is so large that turbulences are absent. Another reason is when haematic hyperviscosity, due to polycythemia consequent to persistent hypoxia, is also present (Kirby and Gillick, 1974), like in our case.

REFERENCES

Eyster, G.E., Weber, W. and McQuillan, W., 1977. Tetralogy of Fallot in a cat. *Journal of the American Veterinary Medical Association*, August 1, **171**(3), 280–282

Feldman, B.V., Zinkl, J.G. and Jain, N.C., 2000. *Schalm's veterinary haematology*, 5th ed., Lippincott Williams & Wilkins, Philadelphia, Pennsylvania USA, 1064–1068

Goodwin, J.K. and Cooper, R.C., 1992. Understanding the pathophysiology of congenital heart defects. *Veterinary Medicine*, July, **87**, 650–668

Kirby, D. and Gillick, A., 1974. Polycythemia and tetralogy of Fallot in a cat. *The Canadian Veterinary Journal*, April, **15**(4), 114–119

Patterson, D.F., Pexieder, T., Schnarr, W.R., Navratil, T. and Alaili, R., 1993. A single major-gene defect underlying cardiac conotruncal malformations interferes with myocardial growth during embryonic development: studies in the CTD line of keeshond dogs. *American Journal of Human Genetics*, February, **52**(2), 388–397

Veterinary Research Communications, 28 (2004) 347–349

Mitral Insufficiency in an Arctic Wolf

C. Guglielmini[1]*, F. Rocconi[1], L. Brugnola[2], C. Vantini[2] and F. Valerio[1]
[1] *Department of Veterinary Clinical Sciences, School of Veterinary Medicine, University of Teramo, Viale Crispi 212, I-64020 Cartecchio (TE), Italy;* [2] *National Wildlife Refuge of Popoli, Corpo Forestale dello Stato, Italy*
* *Correspondence: Dipartimento di Scienze Cliniche Veterinarie – Facoltà di Medicina Veterinaria – Viale Crispi 212, 64020 Teramo, Italy*
E-mail: guglielmini@vet.unite.it

Keywords: wolf, cardiovascular system, echocardiography, valvular disorders

INTRODUCTION

During the second half of last century, the wolf (*Canis lupus* Linnaeus, 1758) was an endangered species in many European countries. In recent decades specific protection programs have resulted in a progressive increase of live wolves in different countries, Italy included. Accordingly, many scientific studies have been carried out to investigate different aspects of wolf ecology. The vast majority of veterinary studies on this wild canid are mainly related to the anaesthetic protocols for its capture and restraint, as well as to its susceptibility to infectious and parasitic diseases. Little is known regarding the prevalence of non-infectious diseases in the wolf and, among them, of cardiovascular disorders.

In the present paper, the clinical, echocardiographic, and echo-Doppler findings of mitral regurgitation in a captive arctic wolf are described.

MATERIALS AND METHODS

A 10-year-old, captive male arctic wolf, housed at the National Wildlife Refuge of Popoli, in central Italy, was examined during the annually scheduled health check. The subject was immobilized with an intramuscular injection of xilazine and ketamine. Anaesthesia was maintained with isofluorane in oxygen after endotracheal intubation. A physical examination, including cardiac auscultation, and blood sampling for complete blood count (CBC) and biochemical profile were carried out. The echocardiographic examination (real-time B-mode and M-mode) and the echo-Doppler examination were conducted using a portable ultrasound machine equipped with a 3.5 MHz mechanical sector transducer and Doppler frequency of 2.5 MHz, with simultaneous electrocardiograms. The standard echocardiographic scan planes described in domestic carnivores (Thomas *et al*., 1993) were employed. Measurements of the cardiac chambers and walls, as well as spectral cardiac Doppler flow profiles were obtained.

RESULTS

On physical examination, a loud systolic murmur (IV/VI grade) heard best over the mitral area was appreciable. Results of CBC and the serum biochemical profile were normal. Two-dimensional echocardiographic examination revealed a mild thickening of the mitral valve leaflets (Fig. 1) associated with a mildly dilated left atrium (left atrial diameter/aortic diameter ratio = 1.8) (Fig. 2).

On echo-Doppler examination, a systolic regurgitant flow across the mitral valve

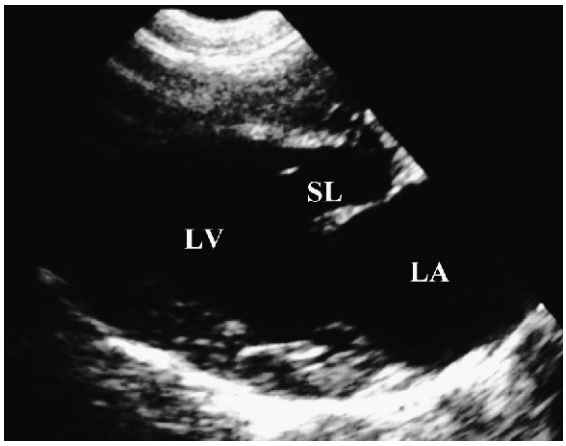


Figure 1. Two-dimensional long-axis echocardiographic image. Note the mild thickening of the septal leaflet (SL) of the mitral valve. LV = left ventricle, LA = left atrium

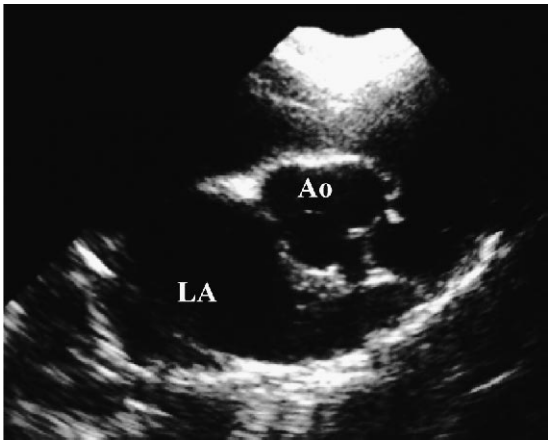


Figure 2. Two-dimensional short-axis echocardiographic image. Note the mildly dilated left atrium (LA) with respect to the aortic root (Ao)

was noted. No other echocardiographic and echo-Doppler abnormalities were found. Thus, mitral regurgitation, most likely due to mitral endocardiosis (ME), was diagnosed.

DISCUSSION

Mitral regurgitation is the most common and clinically important valvular disorder in the domestic dog (Bonagura and Luis Fuentes, 2000). Furthermore, mitral degeneration (endocardiosis) is the most common cardiac disease in dogs (Buchanan, 1992). Aged small and middle-size canine breeds are usually affected (Thrusfield *et al*., 1985; Whitney, 1974), although ME is also diagnosed in large breed dogs (De Madron, 1992; De Madron, 1998). Mild thickening of the mitral valve leaflets, without other appreciable echocardiographic abnormalities, and absence of clinical and clinical pathology evidence of an inflammatory process, strongly suggested chronic degenerative mitral valve disease as the inciting cause of mitral regurgitation in the present case. Mild left atrial enlargement (Rishniw and Erb, 2000) was the only cardiac consequence of mitral insufficiency for the wolf in the present report. Mitral regurgitation was also found in three out of seven aged Italian grey wolves undergoing a diagnostic protocol similar to that of the arctic wolf described in the present paper (Guglielmini *et al*., 2003).

To the best of the author's knowledge, this is the first report of the *in vivo* diagnosis of mitral insufficiency in a wolf. Mitral endocardiosis seems to be a relatively common cardiac disorder in the aged wolf, as for the domestic dog.

REFERENCES

Bonagura, J.D. and Luis Fuentes, V., 2000. Echocardiography. In: S.J. Ettinger and E.C. Feldman (eds), *Textbook of Veterinary Internal Medicine*, 5th edition (WB Saunders, Philadelphia), 834–873

Buchanan, J.W., 1992. Causes and prevalence of cardiovascular disease. In: R.W. Kirk and J.D. Bonagura (eds), *Current Veterinary Therapy, XI: Small Animal Practice* (WB Saunders, Philadelphia), 647–655

De Madron, E., 1992. Primary acquired mitral insufficiency in adult large breed dogs. *Proceedings of the 10th ACVIM Forum*, 608–609

De Madron, E., 1998. Unusual aspects of mitral valve disease in the dog. *Proceedings of the 16th ACVIM Forum*, 116–118

Guglielmini, C., Rocconi, F., Brugnola, L., Valerio, F., Mattei, L. and Boari, A., 2003. Echocardiographic and echo-Doppler examination in the European wolf (*Canis lupus* L., 1758). *Proceedings of the 13th IVRA Congress*, 15

Rishniw, M. and Erb, H.N., 2000. Evaluation of four 2-dimensional echocardiographic methods for assessing left atrial size dogs. *Journal of Veterinary Internal Medicine*, **14**, 429–435

Thomas, W.P., Gaber, C.E., Jacobs, J., Kaplan, P.M., Lombard, C.W., Moïse, N.S. and Moses B.L., 1993. Recommendations for standards in transthoracic two-dimensional echocardiography in the dog and cat. *Journal of Veterinary Internal Medicine,* **7**, 247–252

Thrusfield, M.V., Aitken, C.G.G. and Darke P.G.G., 1985. Observation on breed and sex in relation to canine heart valve incompetence. *Journal of Small Animal Practice,* **26**, 709–717

Whitney, J.C., 1974. Observation on the effect of age on the severity of heart valve lesion in the dog. *Journal of Small Animal Practice*, **15**, 511–522

Veterinary Research Communications, 28 (2004) 351–353

Personal Experiences in the Use of Association Tiletamine/Zolazepam for Anaesthesia of the Green Iguana (*Iguana iguana*)

M. Mauthe von Degerfeld*
Department of Animal Pathology – Faculty of Veterinary Medicine, University of Torino, via Leonardo da Vinci, 44, 10095 Grugliasco (TO), Italy
**Correspondence: Dipartimento di Patologia Animale – Facoltà di Medicina Veterinaria, Via Leonardo da Vinci, 44, 10095 Grugliasco (TO), Italia*
E-mail:mitzy.mauthe@unito.it

Keywords: anaesthesia, green iguana, isoflurane, reptiles, tiletamine/zolazepam

Abbreviations: Z, Zoletil 100®; HR, heart rates; RR, respiratory rates

INTRODUCTION

The green Iguana is one of the most popular reptile pets, so it is very important to find anaesthetic protocols useful for the anaesthetization of these potentially violent patients which are also particularly sensitive to handling stress.

According to the bibliography (Addis *et al*., 1990; Bennet *et al*., 1998; Barten, 1993; Heard *et al*., 2002; Mazzi, 2000; Nevarez *et al*., 2002) reptiles respond to anaesthetic drugs differently from mammals and birds. Injectable agents have long induction times, poor muscle relaxation and rough recovery. Inhalation anaesthesia is not always useful because lots of species can hold their breath even for several minutes or hours.

MATERIALS AND METHODS

Six green iguanas (*Iguana iguana*) of different sexes, ages and weights (mean: 1233.3 ± 251.6 g; range: 1000–1500 g) received a dorsal i.m. injection of a commercial combination of tiletamine/zolazepam (Z), dissolved in the standard way.

Four animals were intubated because diagnostic manipulations or surgery were too long. They inhaled Isoflurane/O_2 by means of a T-Ayre. For endotracheal intubation a shortened defluxion tube made from a "Butterfly" was used and a cat urine catheter was placed in it so it was stiffer to slide. Each iguana was carefully weighed.

Posologies were reduced in the event of serious wasting or dehydration. All animals were checked during anaesthesia and recovery. Heart and respiratory rates were recorded 5 min from inoculation and then at 10 minute intervals throughout the anaesthetic period. These rates were noted at the end of Isoflurane/O_2 use and 10 min

after extubation. The time taken to reach the surgical plane of anaesthesia and the adverse effects during anaesthesia and awakening were recorded. Statistical analysis was performed (mean and range of variation).

RESULTS

Posology – Each iguana received i.m. 10.53 ± 4.2 mg/kg of Z and immediately all animals became excited if not restrained, then anaesthetic effects started and their muscles relaxed a few minutes later. The iguana placed itself in sternal decubitus recumbency then the reptile could be easily handled. Induction time was defined as no response pressing between digits (mean: 6.5 ± 0.7 min) and anaesthesia was considered as achieved when were no righting or toe reflexes. With Isoflurane/O_2 the patients lost their palpebral reflexes after 5 min.

Intubation – Good relaxation of the jaw muscle, even without application of local anaesthetic to the surface of the glottis, facilitated intubation.

Monitoring – Cardiac frequency increased slightly in these animals (Addis *et al*., 1990; Bennet *et al*., 1998); both patients that only received Z and those who received Isoflurane/O_2 had 100 beats/min on average. Respiratory frequencies decreased, their mean values were 10 breaths/min in the first case and 6 in the second one. Anaesthetic concentration: mean $1.8 \pm 0.4\%$ of Isoflurane.

Awakening – The endotracheal tube was removed 12 min from the end of Isoflurane administration. Recovery from anaesthesia was longer than 45 min for some of these patients.

There were no particular side effects.

DISCUSSION

The bibliography regarding reptilias anaesthesia and especially the green iguana is poor. However, according to many authors (Heard *et al*., 2002; Mazzi, 2000; Nevarez *et al*., 2002) inhalation agents are the best in spite of the limitations caused by voluntary apnoea, even if some prefer direct intubation without a pharmacological preventive treatment.

Injectable anaesthetics have some counter indications (longer induction and recovery, less muscle relaxation etc.). It was possible to use Z with posologies indicated by other authors (Addis *et al*., 1990; Barten, 1993) but lower than those reported by Mazzi (2000). This anaesthetic allows a quicker induction and the presence of benzodiazepine gives the muscle relaxation required for handling and orotracheal intubation. No other analgesic is needed as Nevarez *et al*. (2002) suggest, because cyclohexamine provides sufficient action during diagnostic and surgical procedures. Using Zoletil i.m. in the cranial dorsal muscles there is no dosage problem related to

the presence of the renal portal circle and its use is easier than that of other anaesthetics which require an i.v. or intraosseous injection (Bennet *et al*., 1998). The heart rate increase caused by cyclohexamine is useful to avoid bradycardia, a very common side effect when using propofol (Bennet *et al*., 1998). Cyclohexamine also prevents side effects caused by the use of propofol. Zoletil did not cause either inducted apnoea or hypoxia. Orotracheal intubation allows use of a lower concentration of Isoflurane than in anaesthetic chambers or under maintenance using a mask (Addis *et al*., 1990; Barten, 1993). Long recovery times are necessary, not 12 h as Mazzi (2000) because of Ketamine.

Use of Zoletil does not cause excessive excitement in the green iguana, not similarly to effects noted for mammals and birds. These experiences show that this anaesthetic method is valid for anaesthesia of the green iguana.

REFERENCES

Addis, F., Mortellaro, C.M. and Vanosi, G., 1990. Anestesia degli animali "insoliti" – II Anestesia nei rettili. *Summa*, **7**(3), 139–150

Bennett, A.A., Schumacher, J., Hedjazi-Haring, K. and Newell, S.M., 1998. Cardiopulmonary and anesthetic effects of propofol administered intraosseously to green iguana. *Journal American Veterinary Medicine Association*, **212**(1), 93–98

Barten, S.L., 1993. The medical care of iguanas and other common pet lizards. *Veterinary Clinics of North America.: Small Animal Practice*, **23**(6), 1213–1249

Heard D., Fleming G., Lock, B. and Jacobson, E., 2002. *British Small Animal Veterinary Association: Manual of Exotic Pets*, 4th edition (Ed. Meredith, A. and Redrode, S., Gloucester), 223–240

Mazzi, A., 2000. *Elementi di anestesia degli animali esotici e selvatici*. (Ed. Calderoni Edagricole, Bologna), **10**, 73–83

Nevarez, J.G., Pettifer, G.R. and Mitchell, M.A., 2002. *Journal American Veterinary Medicine Association*, **220**(7), 982–985

Veterinary Research Communications, 28 (2004) 355–357

Anticardiolipin Antibodies in Dogs

R. Papini[1]*, O. Pellegrini[2], C. Rossini[1] and G. Guidi[1]
[1] *Department of Veterinary Clinics, Viale delle Piagge 2 – 56124 Pisa – Italy;*
[2] *Practising Veterinarian*
* *Correspondence: Dipartimento di Clinica Veterinaria, Viale delle Piagge 2 – 56124 Pisa – Italy*
E-mail: rpapini@vet.unipi.it

Keywords: anticardiolipin, antibody, antiphospholipid syndrome, dog, Italy

Abbreviations: CL, Cardiolipin; ACA, anti-CL antibodies; APS, antiphospholipid syndrome; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; GPL, IgG/ml units

INTRODUCTION

Cardiolipin (CL) is an acidic phospholipid, first discovered in beef heart tissue but not specific to the heart. It is found both in bacteria and mitochondria of mammals. Mitochondria contain CL as an essential component of the intricate structure of the mitochondrial cristae membrane. CL is more abundant in heart tissue only because of the high concentration of mitochondria in cardiac myocytes. In recent years, anti-CL antibodies (ACA) have been extensively studied in human medicine and their role in antiphospholipid syndrome (APS), a potentially life-threatening autoimmune coagulation disorder, has been well established. Clinical features that should alert the physician to consider APS include recurrent foetal loss, arterial or venous thrombosis, thrombocytopenia and high positivity to the ACA test (Harris *et al*., 1999). However, searching in the literature, we found a lack of investigation on ACA in veterinary medicine. Therefore, we aimed to carry out a preliminary research to verify the presence of IgG ACA levels in dog serum.

MATERIALS AND METHODS

In this preliminary study we tested IgG ACA levels in healthy and diseased dogs. All the dogs tested were randomly selected among those referred to the clinic of our veterinary faculty. There were 116 males and 81 females, ranging from 2 months to 14 years of age, and of different breeds. 134 of them were healthy and were referred to our clinic for regular vaccinations. These animals were used to determine the reference range values. 63 were referred because of a variety of pathologies. These were used to determine IgG ACA levels for diseased dogs. For our purpose, the ELISA technique was used as routinely performed for diagnosis of APS in humans (Tincani *et al*., 1986). Commercially available antidog IgG conjugated with alkaline-phosphatase

(Sigma-Aldrich, S.r.l., Milano, Italy) were used to partially modify the technique for dogs. Optical density was read using a spectrophotometer at 450 nm and was expressed as mean $\pm$ standard deviation of IgG/ml units (GPL).

RESULTS

ACA levels were present for our group of healthy dogs, with a mean value of 5.40 ± 2.66. A high prevalence of ACA has also been documented for populations of healthy adults and children (Cabiedes *et al*., 2002; Mannoussakis *et al*., 1987). Moreover, based on the different levels obtained, dogs with various pathologies could be divided into five groups corresponding to the human model (Harris *et al*., 1987). 18 dogs (group I) had ACA levels within the reference range with values of 3.35 ± 1.73 GPL, 12 (group II) were low-positive with values of 8.59 ± 1.49 GPL, 19 (group III) had moderate values of 22.78 ± 5.65 GPL, 10 (group IV) were high-positive with values of 49.05 ± 11.14 GPL, and lastly 4 (group V) were very high-positive with values of 85.75 ± 9.63 GPL.

DISCUSSION

Diagnosis of APS in humans requires the presence of both clinical and laboratory abnormalities. It can be made when the presence of an elevated ACA level occurs in addition to a thrombotic event associated with vascular thrombosis, thrombocytopenia, and recurrent foetal loss, or with haemolytic anaemia, livedo reticularis, and neurologic disorders (Harris, 1986; Olmstead, 2001; Quintero del Rio, 2002). It is worth noting that at least two of the four dogs in group V had clinical manifestations, history and laboratory abnormalities which would be suggestive of APS for humans. An eight-year-old female boxer was referred because of spondylarthrosis with a history of infertility and recurrent foetal loss; laboratory abnormalities showed serious thrombocytopenia (platelet count $101 \times 10^3/\mu l$; reference range $240–400 \times 10^3/\mu l$) as well as a very high ACA level. A three-year-old male crossbreed was referred because of progressive blindness and retinal thrombosis was diagnosed, together with a very high ACA level. Therefore, our report shows that ACA are present in both healthy and diseased dogs, can be easily detected using the ELISA technique, and can reach very high levels, suggesting a possible role in the pathogenesis of some disorders of dogs. However, at present, it is not possible to affirm that APS can exist in dogs. The present findings are too preliminary and still too limited to allow such a conclusion. Further investigation is certainly needed to clarify the role, if any, of ACA in dogs.

REFERENCES

Cabiedes, J., Trejo-Hernandez, J., Loredo-Abdala, A., Castilla-Serna, L., Lopez-Mendoza, A.T., Cordero-Esperon, H.A., Huerta, M.T., Cabral, A.R. and Alarcon-Segovia, D., 2002. Anti-cardiolipin,

anti-cardiolipin plus bovine, or human beta(2)glycoprotein-I and anti-human beta(2)glycoprotein-I antibodies in a healthy infant population. *Archives of Medical Research*, **33**, 175–179

Harris, E.N., Chan, J.K., Asherson, R.A., Aber, V.R., Gharavi, A.E. and Hughes G.R.V. 1986. Thrombosis, recurrent fetal loss and thrombocytopenia. Predictive value of the anticardiolipin antibody test. *Archives of Internal Medicine*, **146**, 2153–2156

Harris, E.N., Gharavi, A.E., Patel, S.P. and Hughes, G.R., 1987. Evaluation of the anti-cardiolipin antibody test: report of an international workshop held 4 April 1986. *Clinical of Experimental Immunology*, **68**, 215–222

Manoussakis, M.N., Tzioufas, A.G., Silis, M.P., Pange, P.J., Goudevenos, J. and Moutsopulos, H.M., 1987. High prevalence of anti-cardiolipin and other autoantibodies in a healthy elderly population. *Clinical and Experimental Immunology*, **3**, 557–565

Olmstead, J., 2001 A review of antiphospholipid syndrome. *Journal of the American Academy of Nurse Practice*, **13**, 460–463

Quintero Del Rio, A.I., 2002. Antiphospholipid antibodies in pediatrics. *Current Rheumatology Report*, **4**, 387–391

Tincani, A., Balestrieri, G., Allegri, F., Cinquini, M., Vianelli, M., Taglietti, M., Sanmarco, M., Ichikawa, K., Koike, T., Meroni, P. and Boffa, M.C., 2000. Overview on anticardiolipin ELISA standardization. *Journal of Autoimmunity*, **15**, 195–197

Veterinary Research Communications, 28 (2004) 359–362

Quantitation of the Cytokines TNF-α, IL-8 and IL-10 in Bovine Milk Using Real-Time TaqMan® PCR

A. Peli[1]*, A. Luciani,[2], P. De Santis[3], A. Polci[3] and D. Britti[2]
[1] *Department of Veterinary Clinical Sciences, Internal Medicine Section, Faculty of Veterinary Medicine, University of Bologna, Ozzano Emilia, Italy;* [2] *Department of Veterinary Clinical Sciences, Internal Medicine Section, Faculty of Veterinary Medicine, University of Teramo, Italy;* [3] *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy.*
* *Correspondence: Dipartimento Clinico Veterinario, Sezione di Medicina Interna, Facoltà di Medicina Veterinaria, Alma Mater Studiorum – Università di Bologna, Via Tolara di Sopra 50, 40064 – Ozzano dell'Emilia (BO), Italy*
E-mail: apeli@vet.unibo.it

Keywords: dairy cow, mastitis, cytokines, real-time TaqMan® PCR

INTRODUCTION

Interest has recently been increasing as to the role cytokines play in modulating the immune response. However, in the veterinary field, this line of research is still in its infancy. Few studies have been made on the quantification of the cytokines present in dairy milk and these have been conducted only on non-mastitic animals (Leutenegger *et al*., 2000). The evaluation of the cytokine profiles in dairy milk has previously been achieved using the reverse transcriptase-polymerase chain reaction (RT-PCR) method, both for healthy and mastitic cows (Peli *et al*., 2003). In this study, cytokine quantification in dairy milk was determined using the relatively newly developed real-time TaqMan® reverse transcriptase polymerase chain reaction (real-time TaqMan® RT-PCR) method. A comparison was made between healthy cows and animals affected by mastitis, the latter having a high ($> 5.0 \times 10^5$) or a low ($< 1.5 \times 10^5$) number of milk somatic cells as measured by the Somatic Cell Count (SCC) test. All milk samples were analysed for the presence of three cytokines, chosen because of their key role in the immunological regulatory process: IL-8 (chemotactic activity), TNF-α (pro-inflammatory activity) and IL-10 (regulatory/anti-inflammatory activity).

MATERIALS AND METHODS

Milk was collected from 15 Italian Friesian dairy cows at mid-lactation. Two 50 ml aliquots of milk were manually and aseptically collected from each udder quarter. One aliquot was used for the standardised bacteriology and SCC procedures and the

second was used to extract the mRNA coding for the three cytokines TNF-α, IL-8 and IL-10. For each animal only the milk sampled from one of the udder quarters was used, which was selected on the basis of the bacteriological and SCC test results. All milk cells were concentrated by centrifugation at 700G for 10 min at 4°C. The supernatant and the fat layer were discarded and the resulting pellet was washed twice in cold PBS (pH 7.4), and then re-suspended in 250–500 μl of PBS. The mRNA was extracted from a 15 ml starting volume of each milk sample, using the Micro Fast-Track mRNA isolation kit (Invitrogen), and re-suspended in 50 μl of nuclease-free H_2O (Promega). All the extracted mRNAs were kept at −80°C until use. To quantify the expression of the cytokines coding for mRNA in the samples, the real-time TaqMan® PCR method, capable of detecting less than 1 μg of mRNA, was used. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was simultaneously processed in Real-time TaqMan® PCR for each sample, as an endogenous control. This was done to normalise the process for the variability in the number of cells in the original samples, for differences in extraction efficiency, for mRNA degradation in the starting material, and for reverse transcriptase reaction efficiency. The primers and the probe used for the detection and quantification of the IL-10 cytokine were designed by us using sequence data available in GenBank®, and were achieved with the aid of the Primer Express software (PE Applied Biosystems). The forward and reverse primers are located in two adjacent exons, while the probe lies in-between them to hybridise at the junction of the two exons. The primers and probes for the IL-8 and TNF-α cytokines, and for the endogenous control (GAPDH), were obtained from the literature (Leutenegger *et al*., 2000). Each TaqMan® probe was labelled at the 5′ end with fluorochrome FAM (6-carboxyfluorescein) and with fluorochrome TAMRA (6-carboxytetramethyl-rodamine) at the 3′ end (PE Applied Biosystems). The Real-time PCR and reverse transcription of the mRNA target was performed using a "one-step" reaction method, in a final volume of 25 μl. The primers were used at a concentration of 400 nM each, while the probe was used at a concentration of 80 nM, in the reaction buffer (One-Step PCR MasterMix; PE Applied Biosystems), which also contained the reverse transcriptase enzyme and the *AmpliTaq*Gold® enzyme. Finally, 1 μl of extracted mRNA was added to the mixture. The reverse transcription, and real-time TaqMan® PCR, was executed in the ABI Prism® *7700* Sequence Detection System (PE Applied Biosystems), and was done as follows: a 30 min cycle at 48°C, a 10 min cycle at 95°C, then 50 alternating cycles each of 15 s at 95°C, and of 60 s at 60°C. Cytokine quantification was achieved using the Ct (Cycle threshold) comparative method, and is expressed as "n-fold upregulation of cytokine transcription" in relation to a calibrator which is represented by the smallest signal detectable for that specific cytokine.

RESULTS AND CONCLUSIONS

Based upon the results obtained from the bacteriological and SCC tests the Friesians fell into three groups of five animals each: Group A showing an average SCC of

4.097×10^3, and the presence of *Corynebacterium sp.*, *Staphylococcus sp.*, and *Streptococcus sp.* in two, one and two animals, respectively; Group B, showing an average SCC of 74×10^3 and the presence of *E. coli*, and *Streptococcus sp.* in two and three animals respectively; Group C, showing an average SCC of 59×10^3, and no bacteria detected in the milk. The analytical method was able to quantify all the cytokines in all 15 samples examined, and thus confirms the utility of this method for the study of cytokine gene expression in milk. The results, expressed as group mean values of the three cytokines analysed, are shown in Fig. 1. The levels of TNF-α were significantly higher in animals with positive bacteriology (Groups A and B) than in Group C (negative bacteriology), and are not linked to SCC. This result confirms that a bacterial infection, independent of any chemotactic effect, stimulates an increase in

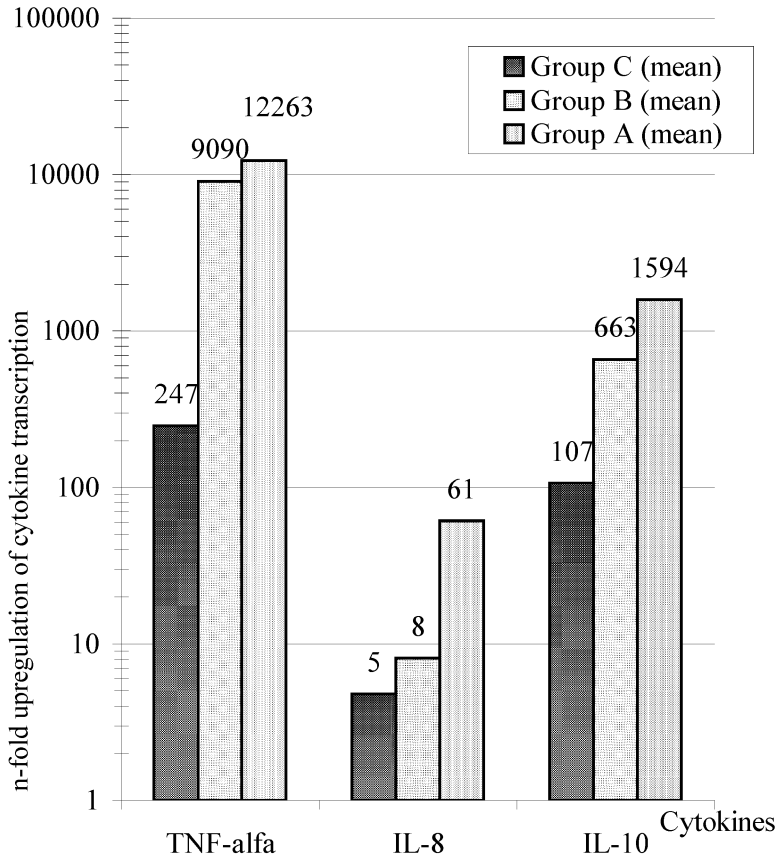


Figure 1. Mean values of the three cytokines analysed in milk cells from groups A, B and C. Cytokine transcription is expressed as the n-fold differences to the calibrator, which was the lowest detectable cytokine signal. Note the semilogarithmic scale

the production of TNF-α (which causes a local inflammatory response). To the contrary, in samples with high SCC (Group A), levels of IL-8 were higher than seen in the low SCC Groups B and C. This confirms the chemotactic role (mostly on the neutrophils) of this cytokine the synthesis of which does not seem to be induced by the presence of bacteria, as is the case for TNF-α, and as deduced from the lower levels of this cytokine detected for Group B (Barber and Yang, 1998). These data confirm recent findings which show that an increase of TNF-α is not necessary to stimulate the synthesis of IL-8 in the mammary gland (Personn *et al*., 2003). As regards IL-10, the results seem to show a correlation with SCC, but the lack of published data on the precise role and origin of this cytokine in the ruminant mammary gland indicates the need for further studies for the explanation of our results. Much research remains to be done on the interactive role of these bacteria and their influence on the quantitative profiles of cytokines in the ruminant udder.

REFERENCES

Barber, M.R. and Yang, T.J., 1998. Chemotactic activities in non-mastitic and mastitic mammary secretions: Presence of Interleukin-8 in mastitic but not non-mastitic secretions. *Clinical and Diagnostic Laboratory Immunology*, **5**, 82–86

Leutenegger, C.M., Alluwaimi, A.M., Smith, W.L., Perani, L. and Cullor, J.S., 2000. Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan PCR. *Veterinary Immunology and Immunopathology*, **77**, 275–287

Peli, A., Scagliarini, A., Britti, D. and Boari, A., 2003. Detection of proinflammatory and regulatory cytokines in bovine millk using RT-PCR. *Veterinary Research Communication*, **27**, Suppl. 1, 779–781

Personn-Waller, K., Colditz, I.G., Lun, S. and Ostensson, K., 2003. Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. *Research in Veterinary Science*, **74**, 31–36

Veterinary Research Communications, 28 (2004) 363–366

Case Report of Leishmaniasis in Four Cats

M.G. Pennisi[1]*, M. Venza[2], S. Reale[3], F. Vitale[3] and S. Lo Giudice[1]
[1] *Department of Medical Veterinary Sciences – Faculty of Veterinary Medicine, University of Messina, Italy;* [2] *Self employer;* [3] *Zooprophylactic Experimental Institute of Sicily "A. Mirri", Palermo, Italy*
* *Correspondence: Dipartimento di Scienze Mediche Veterinarie – Facoltà di Medicina Veterinaria, Polo Universitario dell'Annunziata, 98168 Messina, Sicily, Italy*
E-mail: MariaGrazia.Pennisi @ unime.it

Keywords: cat, leishmaniasis, FIV, therapy

Abbreviations: FIV, Feline immunodeficiency virus; IFAT, Immunofluorescent antibody test; SID, *Semel in die*; SC, Subcutaneous; PO, *per os*; rHuIFNα–2α, Recombinant human α–2α interferon; BUN, Blood urea nitrogen; CFR, Chronic renal failure; PCR, Polymerase chain reaction

INTRODUCTION

The cat is an unusual host of *Leishmania* spp. and only a few cases of leishmaniasis have been reported for this species, mostly in areas where the microorganism and several species of phlebotomine sandflies are endemic (Pennisi, 2002). About twenty clinical cases have been reported in the literature, sometimes described only briefly with regard to both the clinical course and the therapy employed. Because of this, we wish to describe the clinical findings of four cases of feline leishmaniasis observed between December 1997 and May 2001.

MATERIALS AND METHODS

Four domestic cats were involved (three short-haired, one long-haired); two males (cases 2 and 3) and two females (cases 1 and 4). The cats were six (case 3), 10 (case 4) and 14 (case 1) years of age, in case 2 the age was unknown but the subject was an adult cat. All cats came from the Messina district and they were free-roaming. Diagnosis was achieved by microscopic (smears from cutaneous ulcers and cysts, and from lymph node needle-aspiration) and serological investigations (IFAT) with anti-*Leishmania* antibody titres ranging from 640 (cases 1, 2, and 4) to 1280 (case 3). Further confirmation came from PCR and isolation of *Leishmania* spp. in cases 2, 3 and 4. The cats were tested for FIV and FeLV (Duospeed, BVT, La Seyne sur Mer, France), *Toxoplasma gondii* (IFAT), *Bartonella henselae* (IFAT) and given blood tests (complete blood cell count, biochemistry profile, serum protein electrophoresis, urinalysis). Two cats did not undergo specific treatment (cases 1 and 3) because the owner

was not compliant with the drug regimen. Cases 2 and 4 were treated by oral administration. Case 2 was given three different oral treatments without any success: fluconazole (5 mg/kg SID for 2 months), metronidazole (25 mg/kg) and spiramycin (150,000 UI/kg) SID for 35 days and itraconazole (50 mg SID) for 2 months.

Seven months later the owner decided to suspend all treatment drugs. Case 4 was given allopurinol (20 mg/kg SID) for 15 months. Case 4 was a FIV^+ cat also suffering from pancytopenia. On this occasion erythropoietin (50 UI/kg/48 h SC) and ferrous sulphate were administered (50 mg SID PO) for ten weeks and rHuIFNα–2α (30 UI SID topically on the oral mucosa) for 5 months.

The clinical course of three cases was recorded until the death of the subjects. Cases 2 and 3 were euthanised; case 4 is still alive.

RESULTS

At the time of diagnosis the following clinical signs were observed: depression and anorexia (cases 1 and 4), severe weight loss (cases 1 and 4), pale mucous membranes (cases 1 and 4), dehydration (case 1), solitary (case 3) or systemic lymph node enlargement (cases 2 and 4), presence of a small crusty ulcer (case 1), cutaneous bloody cyst (cases 1 and 3), alopecia (case 4), dyspnea (case 1) and hepatomegaly (case 4).

Ophthalmologic examination highlighted signs of a previous uveitis (cases 1 and 2), inactive chorioretinitis (case 3) and acute uveitis (case 4).

Hematological abnormalities were: non-regenerative anaemia (cases 1 and 4), leukopenia (cases 1 and 4), thrombocytopenia (cases 1 and 4), increased BUN (case 1) and increased creatinine (case 1). All cats showed hyperproteinemia and hyperglobulinemia with monoclonal hypergammaglobulinemia.

Cases 1, 3 and 4 were FIV^+; case 2 was positive for Coronavirus (titre 25). Cases 1 (titre 100), 2 (titre 400) and 4 (titre 6400) were IgG positive for *Toxoplasma*, while cases 2 (titre 256) and 3 (titre 256) were IgG positive for *B. henselae*.

Case 1 (FIV^+) was also affected with CRF complicated by non-regenerative anaemia, bronchopneumonia and pyothorax and died 35 days after diagnosis. In cases 2 (unsuccessful treatment) and 3 (no treatment), development of the disease led to the progressive development of CRF with severe non-regenerative anaemia. Euthanasia was performed 22 months (case 2) and 5 years (case 3) after diagnosis. In case 2 CRF was evident 5 months after diagnosis with consequent cachexia and severe anaemia. At the time of euthanasia lymph node enlargement was no longer evident, the specific antibody titre was 320 and serum protein electrophoresis had changed in the preceding month with reduction of hypergammaglobulinemia and development of hyperalfaglobulinemia. Case 3 (no therapy), which developed urinary signs of renal failure three years later, was healthy for about two more years, when the onset of a proliferative stomatitis led to anorexia; at the same time there was the onset of seizures and an irregular bradycardia (108 beats/minute) associated with a 5th degree heart murmur.

The owner opposed any further investigation and asked for euthanasia. The cutaneous lesions, lymph node enlargement, serum protein electrophoresis and antibody titre did not change over the course of the disease, and when the cat was put down the anti-*Leishmania* titre was 640, with positive lymph node needle-aspiration (PCR and microscopy).

Case 4 (treated with allopurinol, erythropoietin, ferrous sulphate and rHuIFNα–2α) showed a rapid improvement with recovery in appetite and vitality within the first two weeks, while a dramatic improvement of the severe haematological data was seen after three weeks. Five months later a weight gain (3 kg) was observed, along with the regrowth of hair, the liver and lymph nodes enlargements disappeared, and the hypergammaglobulinemia and anti-*Leishmania* antibody titre were reduced. The drug regimen was modified and the cat was given allopurinol for ten further months. At the end of the treatment (15 months in all) *Leishmania* serology and PCR were negative, while neutropenia and hypergammaglobulinemia were still present. Two months later the cat exhibited anaemia, leucopenia, hypergammaglobulinemia and positive antibody (titre 80) once again. In the following months the uveitis recurred, anaemia worsened and the IFA titre rose to 1280. Parasites were isolated once again from the submandibular lymph node. At this time allopurinol (20 mg/kg SID) was recommenced, and the treatment remains ongoing.

DISCUSSION

For the first time feline leishmaniasis has been reported in FIV^+ cats (three cases out of four) and treated with oral drugs, which generally meet with greater compliance from the owners, especially for long-term therapy. Clinical signs of feline leishmaniasis are quite similar to those observed for dogs: lymph node enlargement, cutaneous ulcers, alopecia, weight loss, pale mucous membranes, hypergammaglobulinemia and CRF. On the contrary, cutaneous bloody cysts (with evidence of amastigotes inside) have not been reported for canine leishmaniasis. The ocular lesions cannot be considered specific because of the concurrent FIV infection (cases 1, 3 and 4) and a high anti-*Toxoplasma* titre (case 4). Despite the fact that the literature reports the efficacy of fluconazole (Gangneux *et al*., 1999), itraconazole (Gangneux *et al*., 1999) and spiramycin + metronidazole (Gangneux *et al*., 1999; Pennisi *et al*., 2001), these drugs did not work for the FIV^- cat (case 2) which developed CRF. The same happened in case 3 which, in spite of the FIV infection and the lack of therapy, lived for five more years after diagnosis.

Allopurinol was given to cats for the first time and it was well tolerated in case 4 which had a symptomatic (stage IV) FIV infection. The allopurinol treatment was clinically efficient but, as reported for dogs (Baneth, 2002), did not succeed in eradicating the infection.

ACKNOWLEDGEMENTS

The Authors wish to thank Ms. Angela Burrascano and Ms. Giuseppa Di Giovine for their technical assistance.

REFERENCES

Baneth, G., 2002. A review of the treatment of canine leishmaniasis. *Proceedings of the II International Canine Leishmaniasis Forum*, Sevilla, Spain, 15–19

Gangneaux, J.-P., Dullin, M., Sulahian, A., Garin, Y.J.-F. and Derouin, F., 1999. *Antimicrobial Agents and Chemotherapy*, **43**, 172–174

Pennisi, M.G., 2002. A high prevalence of feline leishmaniasis in southern Italy. *Proceedings of the International Canine Leishmaniasis Forum*, Sevilla, Spain, 39–48

Pennisi, M.G., De Majo, M., Masucci, M., Britti, D., Vitale, F. and Del Maso R., 2001. Efficacy of metronidazole-spiramycin combined therapy in the treatment of canine leishmanisis. *Proceedings of Worldleish 2*, Hersonissos, Crete, Greece, 40

Veterinary Research Communications, 28 (2004) 367–369

Blood Pressure Measurements in Dogs and Horses Using the Oscillometric Technique: Personal Observations

F. Porciello, F. Birettoni, M.B. Conti, C. Marinetti, M.T. Antognoni and G. Fruganti
Department of Clinical Sciences – Section of Internal Medicine, Faculty of Veterinary Medicine, University of Perugia
**Correspondence: Dipartimento di Patologia, Diagnostica e Clinica Veterinaria – Sezione di Medicina Interna – Facoltà di Medicina Veterinaria, Università di Perugia, Via S. Costanzo 4, 06126 – Perugia, Italy*
E-mail: fp1@unipg.it

Keywords: arterial blood pressure, dog, horse, oscillometric technique

Abbreviations: Gr, Group

INTRODUCTION

Blood pressure can be evaluated either by using a direct invasive method (arterial puncture) or an indirect noninvasive method (oscillometric or doppler measurement). However, both methods are rarely used in practice: arterial puncture is potentially dangerous and hard to achieve in a non-sedated patient, while the noninvasive techniques are not widely used because of the lack of materials and methods effective enough to obtain proper measurements (Bodey *et al*., 1996; Edwards, 1992). Because of the few references in the bibliography about arterial blood pressure measurement we have focused our attention on the oscillometric technique as it is the easiest to perform in practice and the only method that can be used to assess the diastolic value.

MATERIALS AND METHODS

Arterial blood pressure was assessed using an oscillometric technique in dogs (49) and horses (33) referred to the Section of Internal Medicine of the Department of Pathology, Diagnostic and Veterinary Clinic of the University of Perugia for clinical evaluation. All horses were clinically normal, while dogs were divided into a control group of healthy dogs and a group of patients with diseases that could affect arterial blood pressure. Measurements were performed using Memoprint™, an electronic device product by Medvet, that assesses the diastolic and systolic blood pressure and the mean heart rate. Four cuffs of different size were available, two for large and medium size dogs, one for small dogs and cats and one for horses. For the dog measurements were performed with the patient in sternal or lateral recumbence with the cuff constricting the distal third of the left forelimb, attempting to maintain the

cuff on the same horizontal plane as the heart in order to evaluate the radial artery pulse. At least five measurements were performed for each dog and the weighted mean was then calculated. For horses the cuff was placed around the base of the tail (coccigeal artery) and three measurements and the arithmetical mean were recorded. We divided the animals into 9 groups, ordered as follows: **Gr A**: two-year-old foals, 8 males and 7 females; **Gr B**: 10 Standardbreds, 6 male, 1 neutered male and 3 females; **Gr C**: 8 non-athletic horses, 4 castrated males and 4 females; **Gr D**: 11 clinically healthy dogs, 8 males and 3 females; **Gr E**: 10 dogs affected by chronic renal failure, 5 males and 5 females; **Gr F**: 6 dogs affected by diabetes, 2 males and 4 females; **Gr G**: 7 dogs with heartworm disease, 4 males and 3 females classes II-III; **Gr H**: 9 dogs with chronic mitral regurgitation, 5 males and 4 females, 7 in treatment for congestive heart failure; **Gr I**: 6 dogs affected by atrial fibrillation, 5 males and 1 female, 2 undergoing treatment for arrhythmia.

The statistical significance of the differences between the groups was evaluated using the Mann-Whitney U test. The repeatability of measurement was evaluated by calculating the coefficient of intraoperator variation. We assumed as acceptable a variability of less than 10%.

RESULTS AND DISCUSSION

The repeatability of measurement, expressed in mmHg, was high for the horse ($\Delta S =$ 3.65 and 2.60 for systolic and diastolic pressure respectively with mean values of 113 and 72) and good in the dog ($\Delta S = 11.15$ and 9.01 with mean values of 143 and 79). The pressure measured for different groups of horses was similar (mean systolic pressure = 113 and diastolic = 72). This is interesting if we consider that in human medicine it is well known that athletes' blood pressure is lower than that of normal people and that this decrease in mean arterial pressure can be used as an index of performance (Ellestad, 1987). By the statistical analysis of our results we conclude, on the contrary, that athletic training is not able to modify the arterial pressure for horses; therefore the arterial pressure cannot be used as a performance index. In normal dogs the mean systolic pressure was 143 mmHg and the diastolic pressure was 79 mmHg. These values are not so different from those obtained in other studies using the direct technique (Mishina *et al*., 1997). Also for the dog we did not find any difference between males and females as described in the literature of human and veterinary medicine (Mishina *et al*., 1997). Negative and positive differences, and their significance, between pathological groups and the control group **Gr D** are noted (n.s. = not significative) in Table I.

Arterial blood pressures, both systolic and diastolic, were statistically higher for dogs affected by chronic renal failure and dirofilariasis than control dogs, while for diabetic dogs only the diastolic values were higher. No differences were evident between dogs with cardiac disease, whether undergoing treatment or not, and normal dogs. Our study highlights the clinical usefulness and applicability of blood pressure

TABLE I
Differences in the mean systolic and diastolic pressure observed between pathological groups and the control group for the dog

	Mean systolic pressure	Mean diastolic pressure
Gr D and **Gr E**	Higher in **Gr E** ($p = 0.02$)	Higher in **Gr E** ($p = 0.02$)
Gr D and **Gr F**	n.s.	Higher in **Gr F** ($p = 0.02$)
Gr D and **Gr G**	Higher in **Gr G** ($p = 0.02$)	Higher in **Gr G** ($p = 0.02$)
Gr D and **Gr H**	n.s.	n.s.
Gr D and **Gr I**	n.s.	n.s.

measurement using the oscillometric technique as an easy and reliable diagnostic tool, especially for some common diseases of the dog.

REFERENCES

Bodey, A.R., Michekll, A.R., Bovee, K.C., Buranakurl, C. and Garg, T., 1996. Comparison of direct and indirect (oscillometric) measurements of arterial blood pressure in conscious dogs. *Research in Veterinary Science*, **61**(1), 17–21

Edwards, N.J., 1992. Monitoraggio della pressione sanguigna con metodi non invasivi e trattamento dell'ipertensione, *Veterinaria*, **6**, 7–9

Ellestad, L.H., 1987. *Stress testing*, 3rd, Davis Co

Mishina, M., Watanabe, T. and Fujii, K., 1997. A clinical evaluation of blood pressure through non-invasive measurement using the oscillometric procedure in conscious dogs. *Journal of Veterinary Medicine Science*, **59**, 989–993

Veterinary Research Communications, 28 (2004) 371–375

Swine Ochratoxicosis: Proteomic Investigation of Epatic Bioindicators

P. Roncada[1,2]*, F. Carta[3], M. Zannotti[4], L. Malagutti[4], F. Sciaraffia[4] and G.F. Greppi[1,2]

[1] *Lab. Epigenomica Applicata ISILS Milano;* [2] *Dipartimento di Scienze Cliniche Veterinarie, Milano;* [3] *Porto Conte Ricerche, Alghero;* [4] *Istituto di Zootecnia Generale, Milano*

* *Correspondence: Department of Veterinary Clinical Sciences, Section of Legal Medicine, Faculty of Veterinary Medicine, University of Milan, via Celoria 10, 20133 Milano, Italy*

E-mail: roncadap@unimi.it

Keywords: liver, mycotoxin, ochratoxin, proteomic, swine

Abbreviations: OTA, ochratoxin A; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; DTT, dithiothreitol; IAA, iodocatamide

INTRODUCTION

Ochratoxin A is a naturally occurring mycotoxin produced by *Aspergillus ocraceus*, mainly in tropical regions, and *Penicillum verrucosum* mainly in temperate areas. Ochratoxin A consists of a polyketide-derived dihydroxy-coumarin moiety linked through the 12-carboxy group to phenylalanine (Beretta, 1998). Research in Ochratoxin A (abbreviated as OTA) has increased since 1994, when The International Agency for Research on Cancer classified OTA as a possible human carcinogen, based on sufficient evidence of carcinogenicity in experimental animal studies (IARC, 1994). *P. verrucosum* grows in cereals in cold climates and, as a result, the presence of OTA is larger in Scandinavia and other cool temperate zone areas of Central Europe. It has been suggested that 50% of OTA intake can be attributed to cereal and cereal products. OTA has a potent toxicity and its nephrotoxic and carcinogenic effects in all mammalian species have been demonstrated. OTA's toxic action seems to be more dangerous than that of Aflatoxin (Muller, 2003). Daily intakes of 1 mg/kg BW for 5–6 days can be lethal. OTA mainly affects protein synthesis and subsequently DNA and RNA synthesis. It is a powerful competitive inhibitor on phenylalanine-tRNA ligase, reducing protein synthesis as a result of its inhibitory action. It seems certain that the supply of phenylalanine can reduce OTA's toxicity. The interest in OTA in zootechny regards only monogastric species as ruminants can convert Ochratoxin A into Ochratoxin α which is less toxic (Abarca, 2003). Pigs and chickens are the animals most exposed to the toxicity of OTA. It is well known that OTA plays a major role in the aetiology of nephritis in pigs, causing serious kidney lesions, while

in poultry OTA can cause neuronal anomalies. In addition, daily intake of OTA can reduce Feed Conversion Rate with subsequent lower growth, and it results in a lower efficiency of the immune system; as a result of kidney damage the animals increase their water consumption. The target organ of toxicity in swine is the kidney, but also the liver which has function of detoxification (Beretta, 1998). The aim of the present work was to evaluate the effects of OTA in the diet of growing pigs, concerning liver damage. These effects were evaluated using proteomic methods, monitoring the effects of the administration of OTA on the soluble protein fraction of the liver. The proteomic tools used were high-definition two dimensional electrophoresis coupled with MALDI-TOF mass spectrometry. 64 Large White x Landrace pigs, half of which were castrated males and half females, from 8 litters, with a mean live weight of 41 kg, were divided into 2 groups of 32 animals each and housed in pens each of which held 8 animals. The control group was given a commercial pig feedstuff (Control diet). The treated group was fed the same feedstuff with the addition of 25 μg/kg Ochratoxin A from *Aspergillus ochraceus* (OTA 25 μg/kg), obtained from Sigma-Aldrich s.r.l. Milano. The diets were provided ad libitum and the animals had free access to drinking water. The pigs were individually weighed at the start of the trial and after 119 days, before slaughtering. Data were recorded regarding Live Body Weight (BW) and Average Daily Weight Gain (ADG). Feed intakes of each pen were recorded at the end of the trial to calculate the Feed Efficiency. The liver of four pigs from each group was collected at slaughtering, immediately stored at -20°C and then submitted to proteomic investigation.

MATERIAL AND METHODS

100 mg of liver tissue were homogenised and the soluble proteins were extracted in 1 ml of lysis buffer: 9.5 M urea, 2% CHAPS, 0.8% Pharmalyte 3–10, 1% DTT and 2 mM PMSF in an ice bath (Rabilloud, 2000). The solution was then centrifuged (18000 *g*) and filtered. The samples contained about 10 mg/ml of proteins and the quantity utilized for electrophoresis was about 1 mg. The IPG strips (home made; range 3–10 and 4–7) were reswelled for 6 h (active: sample was directly put into the buffer), 3 h at 30 V and 3 h at 50 V. The total volume of rehydration was 400 mL, 100 mL of sample for each strip. The run was carried out overnight under a layer of paraffin oil at 15°C, under a voltage gradient up to 8000 V (IPG-phor, Amersham Biosciences). After equilibration steps with DTT and IAA, the strips were put onto second-dimension gel, which was a classical SDS-PAGE, 10%T. The analytical staining was performed using silver staining protocol, while the preparative staining for the identification of proteins in mass spectrometry was coomassie blue. The spots of interest were excised with a cutter, de-stained with an acetonitrile/ammonia bicarbonate solution and dehydrated with acetonitrile, then incubation was carried out overnight with trypsin at 37°C. The reaction was stopped at 4°C and the tryptic digests were analyzed using a MALDI-TOF spectrometer (Tof-Spec 2E, Micromass,

Manchester, UK). The comparison of digests was performed in a data bank using a free-on line software, ProFound (www.proteometrics.com).

RESULTS AND CONCLUSIONS

Figures 1 and 2 show two dimensional maps of the soluble protein fraction (liver) of swine (control, left) and added OTA in the diet (right). The matching of the electrophoretograms indicates that there is a greater expression of the α and β chains of haemoglobin in swine undergoing OTA treatment which is probably due to a haemorrhagic status of the liver. In the animals treated with OTA there is an increased expression of different enzymes with related isoforms: carboxypeptidase A, catalase, fumarate-hydratase, phosphatidyl ethanolamine binding protein and inducible NO synthase. These are hepatic enzymes that are indicators of hepatic and energetic

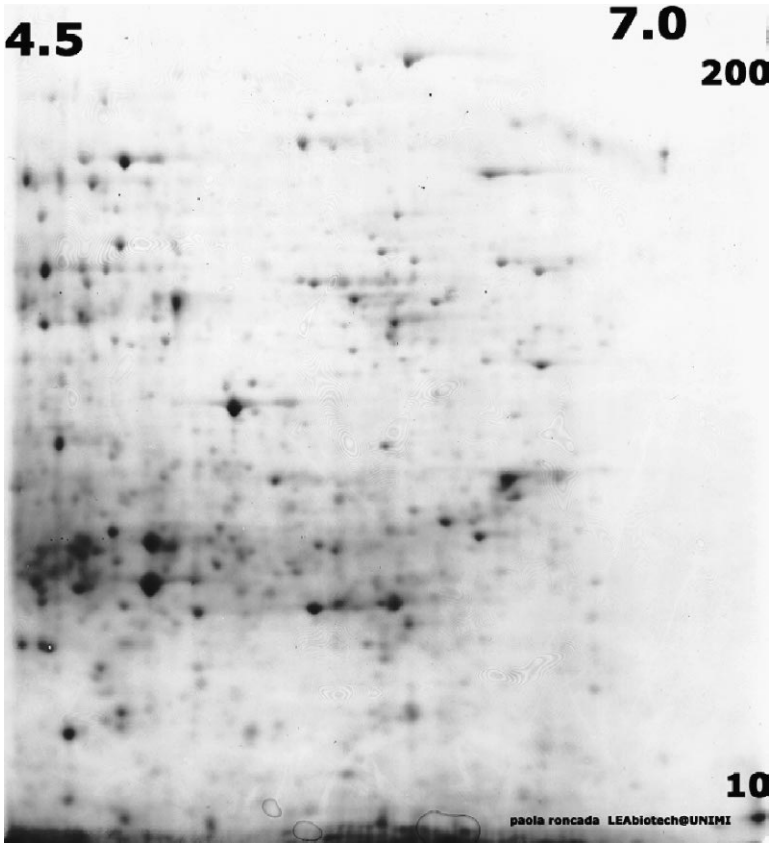


Figure 1. Electrophoretogram of liver tissue of untreated swine (control)

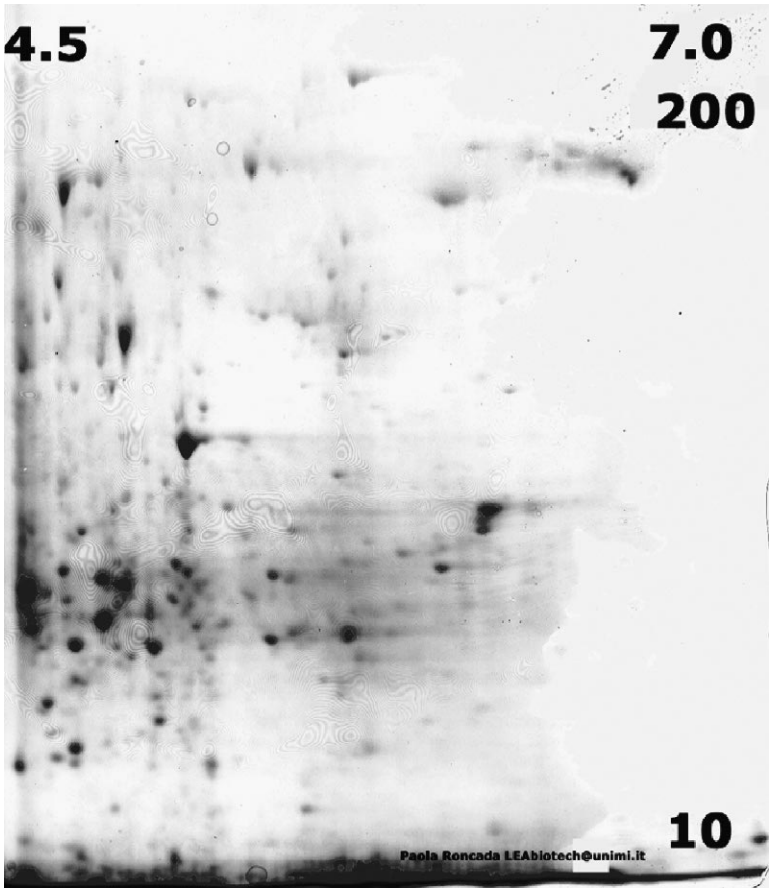


Figure 2. Electrophoretograms of liver tissue of swine treated with OTA

stress. This investigation shows some important differences between the proteins expressed by hepatic tissue under stress conditions; such proteins are not identified with routine analysis. Proteomic investigations are a useful tool in the identification of diagnostic markers.

ACKNOWLEDGMENTS

This work was supported by Progetto PRORICAR (MIUR-Fondo Speciale per lo Sviluppo della Ricerca di Interesse Strategico. U.O. Prof. G.F. Greppi).

REFERENCES

Abarca, M.L. *et al*., 2003. Aspergillus carbonarius as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *Journal of Food Protection*, **66**, 504–506

Beretta, C., 1998. *Tossicologia Veterinaria*, 369–371

Muller, G., Rosner, H., Rohrmann, B., Erler, W., Geschwend, G., Grafe, U., Burkert, B., Moller, U., Diller, R., Sachse, K. and Kohler, H., 2003. Effects of the mycotoxin ochratoxin A and some of its metabolites on the human cell line THP-1. *Toxicology*, **184**, 69–82

International Agency for Reasearch on Cancer, 1994. Ochratoxin A. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, **56**, 489–521. IARC, Lyon, France

Rabilluod, T., 2000. Two dimensional gel electrophoresis and identification methods. *Proteome Research*, 69–71

Veterinary Research Communications, 28 (2004) 377–380

Equine Cushing-like Syndrome: Diagnosis and Therapy in Two Cases

M. Sgorbini[1]*, D. Panzani[1], M. Maccheroni[2] and M. Corazza[1]
[1] *Department of Veterinary Clinical Sciences, Pisa, Italy;* [2] *Endocrinology Laboratory, Azienda Ospedaliera Pisana, Pisa, Italy*
* *Correspondence: Dipartimento di Clinica Veterinaria – viale delle Piagge, 2 – 56100, Pisa, Italia*

Keywords: horse, Cushing-like syndrome, pergolide, ACTH chemiluminescent immunoassay

INTRODUCTION

Equine Cushing-like syndrome or Hyperadrenocorticism is caused by excessive secretion of ACTH by the pituitary gland, secondary bilateral adrenocortical hyperplasia and associated hypercortisolemia. Equine Cushing-like syndrome is due to hyperplasia or adenoma formation of the pituitary *pars intermedia*. A possible cause is the reduction of negative regulation of dopamine on ACTH secretion because of a hypothalamic disease (Dybdal, 1997). Considering this etiopathogenesis a more appropriate description of the disease is probably Pituitary *Pars Intermedia* Dysfunction (PPID) (Schott, 1997). PPID is relatively frequent in old horses (>18 years) with no apparent sex predisposition although authors have suggested that females are afflicted more than males (Boujon *et al*., 1993; Heinrichs, 1990). The characteristic clinical signs include: weight loss secondary to muscle wasting, exercise intolerance, pendulous abdomen, hirsutism and curly hair coat, polyuria and polydipsia, hyperhydrosis, delayed wound healing (Thompson *et al*., 1995) and bulging supraorbital fat pads as a result of fat redistribution.

Less frequent symptoms are skin infections, urinary tract infections, gingivitis, periodontal infections, bronchopneumonia and chronic recurrent laminitis (Hillyer *et al*. 1992; Reed, 1998). Persistent hypercortisolism induces insulin resistance and associated glucose intolerance, thus hyperglycemia and hyperinsulinemia are possible (Beech e Garcia, 1985; Beech, 1987; Reed, 1998). Furthermore, infertility seems to be a common consequence of releasing inhibition of gonadothropic hormones (Love, 1993).

MATERIALS AND METHODS

Case 1

18-years old, mare, Italian saddle horse.

Clinical signs

The horse suffered from chronic recurrent laminitis and a one year history of infertility as well as progressive hirsutism, hyperidrosis, pendulous abdomen, lethargy, polyuria and polydipsia (90 L of water every day) and several months of anorexia. At the time of the visit the subject was also febrile (39.8°C) and the respiratory rate was slightly increased (30 min). Chest auscultation demonstrated harshness of expiratory sounds and inspiratory crackles, particularly in antero-ventral areas. Anoestrus was confirmed by a routine gynecological examination.

Haematology, biochemical profile and urinalysis

Hyperglycaemia (197.7 mg/dl, normal range 62–134 mg/dl) (Eades e Bounous, 1997) and hypostenuria (SG 1002) were the only abnormalities detected. A presumptive diagnosis of PPID was made and a ACTH stimulation test was carried out using esacetate tetracosaetide (Synacthen®, Novartis Farma) at the dose of 100 IU/kg IV. Basal and two hours post-ACTH cortisol plasma concentrations (RIA method, DPC, USA) were 146 nmol/l (normal range: 25–155 nmol/l) and 366 nmol/l (normal range: <400 nmol/l), respectively. ACTH (chemiluminescent enzyme immunoassay, DPC®, USA) and insulin (chemiluminescent enzyme immunoassay, DPC®, USA) plasma concentrations were also measured and were 155 pg/ml (normal range: ≤ 35 pg/ml – Perkins *et al*., 2002) and 241 mUI/ml (normal range: 5.40–36.00 mUI/ml), respectively (Beech e Garcia, 1985; Beech, 1987; Reed, 1998).

In order to establish the normal range of equine ACTH plasma concentration we analysed plasma samples obtained from 5 healthy horses ranging from 17 to 22 years of age. Our range was 13.4 ± 2.23 pg/ml, basically similar to the values reported by others (Couetil *et al*., 1996; Perkins *et al*., 2002). The ACTH and insulin plasma concentrations were dramatically above normal values and were compatible with the diagnosis of PPID.

Treatment

The horse was treated with a dopamine agonist: pergolide mesylate (Nopar®, Lilly, Florence, Italy) initially administered PO at a low-dose: 0.5 mg/24 h to avoid adverse effects of the drug, and gradually increased by 0.5 mg/24 h every 3 days to a final dosage of 3 mg/24 h (Munoz *et al*., 1996). Antibiotics were administered to treat the respiratory problems.

Follow-up

Most of the clinical signs (lethargy, anorexia, PU/PD) resolved in about 6–7 weeks and ovarian activity in 8 weeks as previously observed by others (Reed, 1998). ACTH

plasma concentration was monitored after 45 days and 14 months and was 26.8 pg/ml and 27.7 pg/ml, respectively. At the current time the mare is still in good health and has normal ovarian activity.

Case 2

25-years old mare, French saddle horse.

Clinical signs

The history reported recurrent laminitis lasting several months, hirsutism, pendulous abdomen, bulging supraorbital fat pads and a recent severe progressive polyuria and polydypsia (120 L of water every day).

Haematology, biochemical profile and urinalysis

The haematology and biochemical profile were unremarkable and urinalysis showed hypostenuria (SG 1007). A presumptive diagnosis of PPID was made on the basis of characteristic clinical signs. A definitive diagnosis of PPID was made by measuring the ACTH plasma concentration: the value was found to be more than twenty fold above the normal range: 370 pg/ml, while the insulin plasma concentration was within the normal range: 15.5 mUI/ml.

Treatment

The mare was treated with pergolide mesylate using the same protocol reported before. The definitive dosage was increased up to 4 mg/24 h after one month of therapy because no improvement was observed after four weeks.

Follow-up

Clinical signs ameliorated after about six weeks despite a slight persistent PU/PD. The ACTH plasma concentration was monitored after two and four months and was 91.9 pg/ml and 59.1 pg/ml, respectively. At the current time the mare is still in good health, despite a slight persistent PU/PD.

CONCLUSIONS

Equine PPID is a frequent disease in old horses, although many presumptive diagnoses are probably not confirmed because of the owners' poor compliance and sometimes due to the limited number of laboratories that perform horse ACTH plasma concentration analysis. In the reported cases PPID was suspected at the time of the

visit due to the presence of most of the classical clinical signs of the disease. The definitive diagnosis was strongly supported by the increased ACTH plasma concentration found above >35 pg/ml which is recognised as the critical value with a sensitivity of 100% (Perkins *et al*., 2002).

In contrast with the ACTH-stimulation test and dexamethasone suppression test, only a single blood sample is necessary to measure the basal ACTH concentration, which seems to be more practical and time saving.

Pergolide mesylate treatment was successful for both horses and no side effects were noticed even though anorexia, sweating, dyspnoea, dry mouth, diarrhoea, colic and laminitis are reported as a consequence of higher doses of this drug (Beech, 1999; Munoz *et al*., 1996; Van der Kolk *et al*., 1997). Furthermore, the restoration of fertility in "case 1" permitted successful use of the mare as embryo donor. In both cases the improvement of clinical signs seemed to be associated with reduced levels of ACTH plasma concentration that for the "case 1" mare returned within the normal range.

In conclusion, the ACTH chemiluminescent immunoassay provides a good opportunity for veterinarians to confirm the suspicion of PPID and pergolide is the first drug of choice for its treatment, especially considering that only 1–4 tablets/day have to be given.

REFERENCES

Beech, J., 1987. Evaluation of thyroid, adrenal and pituitary function. *Vet. Clin. North Am. Equine Pract*., **3**, 649–661

Beech, J., 1999. Disease of the pituitary gland. In: Colahan, P.T., Merritt, A.M., Moore, J.N. and Mayhew I.G. (eds), *Equine Medicine and Surgery*, 5th edn. Mosby Inc., St. Louis, pp. 1951–1956

Beech J. and Garcia, M.C., 1985. Hormonal response to Thyrotropin-releasing hormone in healthy horses and horses with pituitary adenoma. *Am. J. Vet. Res*., **46**, 1941–1943

Boujon, C.E., Bestetti, G.E., Meier H.P. *et al*., 1993. Equine pituitary adenoma: a functional and morphological study. *J. Comp. Path*., **109**, 163–178

Couetil, L., Paradis, M.R. and Knoll, J., 1996. Plasma adrenocorticotropin concentration in healthy horses and horses with clinical signs of hyperadrenocorticism. *J. Vet. Intern. Med*., **10**(1), 1–6

Dybdal, N., 1997. Pituitary Pars Intermedia Dysfunction (Equine Cushing's like Disease). In: Robinson NE ed: *Current Therapy in Equine Medicine*, 4th edn. WB Saunders Co., Philadelphia, pp. 499–501

Eades, S.C. and Bounous, D.I., 1997. *Laboratory Profiles of Equine Diseases*. Mosby-Year Book, Inc., St. Louis

Hillyer, M.H., Taylor, F.G.R., Mair, T.S. *et al*, 1992. Equine pituitary tumors. *Eq. Vet. Educ*., **4**, 131–135

Love, S., 1993. Equine Cushing's disease. *Br. Vet. J*., **149**, 139–153

Munoz, M.C., Doreste, F., Ferrer, O., Gonzales, J. and Montoya, J.A., 1996. Pergolide treatment for Cushing's syndrome in a horse. *Vet. Rec*., **139**, 41–43

Perkins, G.A., Lamb, S., Erb, H.N., Schanbacher, B., Nydam, D.V. and Divers, T.J., 2002. Plasma adrenocorticotropin (ACTH) concentrations and clinical response in horses treated for equine Cushing's disease with cyproheptadine or pergolide. *Eq. Vet. J*., **34**(7), 679–685

Reed, S.M., 1998. Pituitary Adenomas: Equine Cushing's Diseases. In: Reed, S.M. and Bayly W.M. (eds), *Equine Internal Medicine*. W.B. Saunders Co., Philadelphia, pp. 912–915

Schott, H.C., Coursen, C.L., Eberhart S.W. *et al*., 2001. The Michigan Cushing's project. In: *Proc. 47th Ann. Conv. Am. Ass. Eq. Pract*., pp. 22–24

Thompson, J.C., Ellison, R., Gillett, R. and Badcoe, L., 1995. Problems in the diagnosis of pituitary adenoma (Cushing's syndrome) in horses. *New Zealand Vet. J*., **43**, 79–82

Van der Kolk, J.H., 1997. Equine Cushing's disease. *Eq. Vet. Educ*., **9**(4), 209–214

Veterinary Research Communications, 28 (2004) 381–384

Veterinary Case History: Forensic and Medical Legal Aspects

G. Signorini[1], G. Biagi[2]*, E. Luchetti[2], G. Cardini[2] and R. Bernocchi[3]
[1] *Specialisation School in "Law and Veterinary Legislation", Faculty of Veterinary Medicine, University of Parma, Italy;* [2] *Department of Veterinary Medicine, University of Pisa, Italy;* [3] *Department of Medical and Forensic Science, University of Parma, Italy*
**Correspondence: Dipartimento di Clinica Veterinaria – Viale delle Piagge, 2 – 56124 Pisa, Italy*
E-mail: biagi@vet.unipi.it

Keywords: veterinary case history

INTRODUCTION

Veterinary case histories exist to document the condition of the animal at the time of the visit and during the course of any illness; they furthermore constitute a particularly useful element in the case of subsequent or periodic controls, allowing the veterinary doctor to access data from previous clinical notes, and on the basis of these to be able to make more useful diagnoses. Since this official record is able to provide an accurate picture of the clinical situation of the animal, the choices made by the doctor and the evolution of the illness, on one hand it constitutes an important source of information for historical health research and the aims of scientific research and statistical analysis, and on the other hand allows any parties suffering damage to reconstruct and evaluate the behaviour of the veterinary doctor and ascertain any professional responsibility. In order to fulfil these tasks correctly, it is considered that case histories must contain at least the following essential information, naturally in addition to the identification data concerning the structure and the veterinary doctor responsible for the case: a) general details of the animal's owner; b) species, race, sex and age of the animal; c) reason for the visit or hospitalisation; d) anamnesis; e) clinical inspection and general controls carried out, and their results; f) collateral exams carried out; g) diagnosis; h) prognosis; i) therapy; j) course of the illness.

LEGISLATION

From a regulative point of view, there are no specific instructions on how to keep veterinary case histories. Although concise, some guidelines can however be found in the instructions governing human medicine. In particular, article 35 of the Italian Ministerial Law (D.M.) of the 27th of June 1986 – determining the requirements for private clinics – sets out that a case history must be completed for every patient, from which the following should be documented: "complete personal data, diagnosis on

arrival, family and personal anamnesis, objective examination, laboratory and specialist examinations, diagnosis, therapy, results and after-effects". This law also foresees that "... the regular updating of the case history during hospitalisation is the responsibility of the chief consultant or head of service, while the filing and issue of copies to all those who are entitled to receive them is the responsibility of the medical director". Clearly the above regulation is designed for the management of case histories in the field of human medicine and, more particularly, in the case of private clinics; however the rules contained in the law also appear to be a useful model for use in veterinary practice, providing helpful indications for correct and suitable behaviour. In law, the function of case histories is described as follows: they are "a diary of the course of an illness and other relevant clinical factors"(1) and "certify the therapy undertaken"(2). More particularly, it states that such records "confirm their function as a diary, and the facts must be written down as they happen"(3). Concerning the content, it is specified that "in the records the diagnosis, progression of the illness and therapies administered on each occasion are noted"(4), "as well as any operations carried out, the results of therapy and the length of the patient's stay in hospital"(5). It follows that, above all in university clinics and veterinary hospitals, where it is almost inevitable that such notes are made by more than one person, it is good practice that each note be initialled by the person making it.

LEGAL ASPECTS

Concerning the legal nature of case histories, and in particular whether they should be considered a public or private document, and whether their contents should be given a privileged legal validity as evidence or not, contrasting views can be found in existing legal doctrine or, in any case, views that do not help the operator to clarify his ideas on this issue. Concerning this matter Piccini (1993) states: "The legal nature of case histories has not yet been firmly defined, meaning whether they should be considered a real public deed that can be used as privileged evidence, or as mere hospital documents with technical and medical relevance, or as documents that can be compared to administrative certification"(6). Pugliatti (1999), on the other hand, more peremptorily states that "the value of the case history as an official record is indisputable"(7).

We feel that the issue of the legal nature of case histories and the legal validity of their contents as evidence is a very delicate matter, and one which should be handled starting from the general meaning of the term *official records*, and the value of these records as evidence. Article 2699 of the Italian Civil Code defines an official record as "the document drawn up in the required formal manner, by a notary or other Public Official (P.O.) authorised to officially certify the place in which the document was made". The following article (2700) then specifies the validity of the document, stating that the "official record constitutes full evidence unless subject to a summons challenging its origin by the P.O who has drawn it up, as well as the declarations from the

parties and other events that the P.O. declares to have taken place in his presence, or to have been carried out by him". Of particular interest to this study, an official record is deemed to be any document drawn up by the P.O. for any purposes inherent to his functions and this even includes purely internal records drawn up with the aim of documenting matters relating to the activities he carries out and the regularity of the administrative operations for which he is responsible [8].

As far as clinical records are concerned, the Supreme Court has deemed that they "must be considered an official record, as they constitute the execution of certificatory power and are part of the public nature of the medical activity to which they refer, not only the case histories kept by a public hospital but also, by virtue of the delegation of public functions conferred upon private institutes by the national health service, records kept by a private clinic that has an arrangement with the national service (specifically on the subject of ideological forgery of documents committed by doctors in medical records obtained from private clinics)"[9]. Having therefore established, albeit briefly, what should be considered an official record, and when a veterinary case history can be considered official, we now need to examine how, and which parts of, an official record (and more particularly a case history) can be considered privileged evidence (as established in the mentioned article 2700 of the Italian Civil Code), meaning that it takes precedence over other sources of evidence. On this matter, legal doctrine deems that privileged evidence cannot concern the whole of an official record, but needs to be reserved and limited to only some of its parts, and more specifically: a) to the declarations by the parties and other events that the P.O. declares to have taken place in his presence; b) to the actions that the P.O. declares to have carried out personally. As far as the remaining parts are concerned, opinions (or judgements) expressed by the P.O. in the document, these cannot be considered privileged evidence and therefore these declarations may be proved to the contrary [10].

CONCLUSIONS

By applying the general concepts outlined above on this subject, we may conclude, in the context of case histories drawn up for example in a university clinic, that privileged evidence may be considered the date and the checks carried out, but not the diagnosis, prognosis and any other matter conditioned by the doctor's own evaluation. The Supreme Court has in fact clarified that "case histories drawn by a doctor in a public hospital cannot, for this matter alone, completely be considered an official document with validity as privileged evidence, as this particular validity as evidence must be limited to their certification by the P.O. and to the events the P.O. declares to have taken place in his presence or to actions that have been carried out by him"[11].

REFERENCES

1. Cass. Pen. Sez. V 10/01/1987 in Cass. Pen. 1987, 825
2. Cass. Pen. Sez. V 08/02/1990, in Cass. Pen. 1991, I, 1771

3. Cass. Pen. Sez. V 21/04/1983 in Cass. Pen. 1984, 2185
4. Cass. Sez. V 05/06/1971 n.563
5. Cass. Sez. VI 24/10/1975 n. 9872
6. Piccini, C., 1993. *Istituzioni di Medicina Legale e delle Assicurazioni*, Milano
7. Pugliatti, G., 1999. *Trattato di Medicina Legale e Scienze Affini*, a cura di Giusti, G., Vol. V, Padova
8. Cass. 25/09/1980 in Giur. It. 82, II, 72
9. Cass. Pen. S.U. 11/07/1992 in Giust. Pen., 1993, II, 65, 301
10. Antolisei, F., 1995. *Manuale di Diritto Penale*, p.s., II, Milano
11. Cass. Pen. Sez. V, 24/10/1980 in Cass. Pen. 1982, 470

Veterinary Research Communications, 28 (2004) 385–388

Serum Values of Cardiac Troponin-T in Normal and Cardiomyopathic Dogs

A. Tarducci*, O. Abate, M. Borgarelli, A. Borrelli, R. Zanatta and A. Cagnasso
Department of Animal Pathology, Faculty of Veterinary Medicine, University of Torino, Grugliasco (To), Italy
**Correspondence: Dipartimento di Patologia Animale – Facoltà di Medicina Veterinaria, Via L. da Vinci, 44 – 10095 Grugliasco (Torino) – Italy*
E-mail: alberto.tarducci@unito.it

Keywords: troponin T, dog, heart, dilated cardiomyopathy, ventricular hypokinesis

INTRODUCTION

Together with tropomyosin, the troponin complex participates in the contraction of skeletal and cardiac muscles. Troponin is composed of three subunits, namely troponin C (TnC), troponin I (TnI), and troponin T (TnT), which binds tropomyosin. There are three different isoforms of TnT and TnI, two of which are specific for skeletal muscle, while the third is specific for the myocardium (cTnT and cTnI). As the homology between troponins is about 95% among mammals, commercial diagnostic kits designed for use in humans also provide excellent results in other animals. (O'Brien *et al*., 1997).

In human medicine, the measurement of cTnT or cTnI levels is now used together with measurement of the levels of creatine kinase (CK) and lactate dehydrogenase (LDH) and their isozymes for assessment of myocardial damage. For diagnostic purposes, one advantage is that these proteins are normally absent in blood in subjects without myocardial damage. Even minimal increases in the serum levels of these proteins are indicative of myocardial damage and, in the case of cTnT, it is detectable in serum from 2 h to 14 days following the onset of myocardial damage. Moreover, in contrast to the isozymes of CK and LDH, the results are not influenced by use of different clinical samples (hemolytic, lipemic, bile samples). The degree of increase in the level of cTnT is relatively proportional to the degree of myocardial damage, with prognostic implications as well. At the same time, the extreme sensitivity of this technique also allows for its use in other nonischemic pathologies including chronic cardiac insufficiency, pulmonary emboli, acute myocarditis, systemic hypertension, arrhythmias, and toxicity from doxorubicin (De Francesco, 2002). In veterinary medicine, O'Brien *et al.* (1997) have demonstrated the utility of measuring the levels of cTnT in the diagnosis of experimentally induced myocardial damage.

MATERIALS AND METHODS

cTnT levels were determined using an immunochemical test developed for humans using whole, heparinized venal blood (Cardiac Reader, Roche) within 2 h of blood

collection. Values of cTnT less than 0.05 were considered negative (expressed in ng/ml). Values of cTnT between 0.05–0.1, 0.1–2, and > 2 were considered as low, intermediate, and high, respectively. The cTnT levels were measured for a control group of 20 dogs that were judged as healthy on the basis of history and objective clinical examination with particular attention to the cardiovascular system. This group of animals comprised subjects with different races and gender with an age between 1 to 12 years (mean 6.1). Another group of 30 animals was defined as cardiomyopathic on the basis of clinical and instrumental examination (e.g., echocardiograph and ecoDoppler, radiography and EKG). These all had cardiac insufficiencies between 1b and 3a, according to the ISACHC classification. On the basis of echocardiographic examination, the myocardiopathic group was further divided into two subgroups: non-hypokinetic myocardiopathic (12 dogs aged between 3 months and 16 years, mean 7.5) and hypokinetic myocardiopathic (global or segmental hypokinetic; 18 dogs aged between 7 months and 15 years, mean 6.0). Statistical analysis using Fisher's test was employed to evaluate the correlation between a positive cTnT test and cardiac insufficiency, as well as ventricular kinetics. The aim of the present work was to determine the value of cTnT for healthy dogs and for dogs affected with different cardiac pathologies, evaluating eventual correlations between cTnT levels, the ISACHC classification for cardiac insufficiency, the index of systolic volume (ESV-I), and ventricular kinetics.

RESULTS

The control group of healthy dogs had values of serum cTnT that were below detectable levels (i.e., < 0.05 ng/ml). Ten of the 30 myocardiopathic dogs (33%) tested positive for cTnT. However, there was no significant correlation between a positive result in the cTnT assay and the type of cardiac insufficiency or with the ESV-I. In the non-hypokinetic group, only one of the 12 dogs (8.3%) which had an intermediate level of cTnT was positive. In the hypokinetic myocardiopathic group, 9 out of 18 animals tested positive, three with low levels of cTnT, four with intermediate levels, and two with high levels. The probability of a positive cTnT value was significantly higher for hypokinetic animals with respect to non-hypokinetic dogs (Fisher's exact test, $\mathrm{OR} = 10$, $p < 0.05$).

It was possible to carry out a second examination for 4 out of 10 dogs at follow-up visits during the course of therapy. In three animals, the cTnT level was below detectable levels by the second control visit and was associated with an improvement in general clinical conditions in all cases. The remaining dog, diagnosed with dilative myocardiopathy, had levels of cTnT that had increased over a two week interval, although they still fell in the intermediate value category. During this period, the dog's conditions progressively worsened in spite of therapy and the animal died three days after the last visit. For the three dogs with dilative myocardiopathy, the value of cTnT was below detection limits.

DISCUSSION

The absence of detectable cTnT (>0.05 ng/ml) in healthy dogs confirms the utility of this assay in assessing acute myocardial damage, similar to that reported both for both human and veterinary subjects (De Francesco, 2002). The results of the present work indicate the usefulness of the cTnT test for the identification of cardiopathic subjects for whom disease is associated with myocardial damage. Furthermore, our data are also interesting in relation to the lack of correlation between a positive cTnT result and the degree of systolic insufficiency. In addition, the observation that only 50% of the hypokinetic group was positive for cTnT suggests that the assay may have particular utility in discriminating animals with myocardial damage in course from those in which hypokinesis is most likely indicative of a previous injury. On the other hand, the positive test results observed in non-hypokinetic, myocardiopathic subjects implies that the measurement of cTnT is a diagnostic tool that is valuable for the identification of myocardial damage, even in animals that would not otherwise be suspected to have myocardial damage on the basis of clinical and instrumental examination.

The determination of cTNT during the follow-up period may also have prognostic implications. During this period, clinical improvement was seen in all three 3 cases with negative cTnT, while in the subject for whom increased levels of cTnT were observed, clinical deterioration was noted and resulted in sudden death. Lastly, for the three dogs with dilative myocardiopathy serum cTnT was not detected. This may be explained by the low concentrations of troponin T at the myocardial level, as reported by O'Brien (1997) for Dobermans affected with idiopathic dilative myocardiopathy. Our results are in contrast to those found for humans, in which a relatively high percentage of patients were found to be positive for cTnT during the course of dilative myocardiopathy. It has been reported that the return to normal levels of cTnT following therapy also has a beneficial impact on prognosis, with survival times that are significantly higher (Sato *et al*., 2001). However, in the present study the number of animals with dilative myocardiopathy, all with negative cTnT, is too small to make any conclusions in this regard.

ACKNOWLEDGEMENTS

This work was supported by grants from Italian MURST.

REFERENCES

De Francesco, T.C., Atkins, C.E., Keene, B.W., Coats, J.R. and Hauck, M.L., 2002. Prospective clinical evaluation of serum cardiac troponin T in dogs admitted to a veterinary teaching hospital. *Journal of Veterinary Internal Medicine*, **16**, 553–557

O'Brien, P.J., 1997. Deficiencies of myocardial troponin-T and creatine kinase MB isoenzyme in dogs with idiopathic dilated cardiomyopathy. *American Journal Veterinary Research*, **58**, 11–16

O'Brien P.J. Dameron, G.W., Beck, M.L., Kang, Y.J., Erickson, B.K., Di Battista, T.H., Miller, K.E., Jackson, K.N. and Mittelstadt, S., 1997. Cardiac troponin T is a sensitive, specific biomarker of cardiac injury in laboratory animals. *Laboratory Animal Science*, **47**, 486–495

Sato, Y., Yamada, T., Taniguchi, R., Nagai, K., Makiyama, T., Okada, H., Kataoka, K., Ito, H., Matsumori, A., Sasayama, S. and Takatsu, Y., 2001. Persistently increased serum concentrations of cardiac Troponin T in patients with idiopathic dilated cardiomyopathy are predictive of adverse outcomes. *Circulation*, **103**, 369–374

Veterinary Research Communications, 28 (2004) 389–391

Postural Pattern Alterations in Orthopaedics and Neurological Canine Patients: Postural Evaluation and Postural Rehabilitation Techniques

C. Vallani*, C. Carcano, G. Piccolo, V. Galardi, P. Longhi, R. Padovano and V. Cantarini
Physiotherapy Unit, Crollalanza Clinic, Milan, Italy
**Correspondence: Sezione di Fisioterapia Veterinaria, Clinica Veterinaria Crollalanza, Via Crollalanza 9, 20100 Milano MI, Italy*
E-mail: vallani@maganet.net

Keywords: dog, rehabilitation, postural pattern

Abbreviations: M, male; F, female; y, year/s; m, month/s; d, day/s; SC, body pattern; PR + xd, beginning of rehabilitation program × days after surgery; F, phase

INTRODUCTION

Physiotherapeutic analysis, including postural evaluation of the patient, is very important in rehabilitative medicine (Basaglia, 2000). Patients with locomotory problems often present altered body and movement patterns. Body Pattern (SC) is defined as: "the sensation that everyone has of their body position in space and of its movement, formed on the basis of proprioceptive information integrated by visual afferences" (Buzzi *et al*., 1996). In physiology this concept is defined as kinaesthesia (Mountcastle, 1977) or somatic sensation (Ruch, 1976). Posture is defined as the position the body adopts in space by means of the tonic activity of muscles acting against gravity (Villeneuve, 1996). Modifications of this body pattern happen, for example, when there is pain in the locomotory apparatus or elsewhere, which leads to a reduction of the range of the movements, to anthalgic and compensatory postures and to substitutive movements or "tricks". This situation induces incorrect motory patterns and new body representations which are memorised by the nervous system. The aim of "neuromotory reprogramming" is to re-establish the normal body and global kinetic patterns by means of active proprioceptor stimulation and suitable postural exercises. In this paper we wish to present a rehabilitating postural methodology, in which global postural exercises, both static and dynamic, are added to the normal rehabilitation program. The exercise technique needs to be "global" as in nature there is no such thing as an isolated single muscle contraction (muscular chains mechanism), as the Nervous System regulates movement as a whole (Souchard, 1994).

MATERIALS AND METHODS

Four clinical cases are reported as examples.

Case 1 = Male (M), 4 years (y), diagnosis: Hansen 1 disk rupture in T13-L1; haemilaminectomy 3 days after the appearance of para-paresis; beginning of rehabilitation program 14 days after surgery (PR + 14d). **Case 2** = F6y, lumbar disk rupture, no surgery; beginning of rehabilitation program 60 days after the appearance of the symptoms. **Case 3** = F7y, multiple pelvis fracture. Reconstructive surgery using bone plates 4 days after trauma, PR + 35d. **Case 4** = F6m, bilateral hip dysplasia. Bilateral triple pelvic osteotomy (TPO) 30 days after the appearance of symptoms, PR + 20. After examining the postural and body pattern alterations, we made the patients perform postural exercises which led the patient to regain the ability to stand on all fours with the correct body pattern. Moreover, these exercises aimed to strengthen the so-called postural or gravity-opposing muscles, to regain normal mobility and to enhance muscle strength and coordination. Coordination, static-dynamic and functional balance exercises are performed mimicking normal movements. They are performed very slowly to facilitate memorisation through repetition, following the action-repetition principle of rehabilitative medicine, always taking care to make the patient always adopt a correct postural and motory pattern. Thus, during the working session postural errors are continuously corrected and neurophysiological responses are activated by following simple and instinctual postural and motory patterns (sit/stand up). Several working phases can be identified: a phase in which the dog lies on one side, another in which the dog is sitting in a "sphinx" or a "sit dog" position, another in which it stands on all fours, and a locomotion phase.

RESULTS AND DISCUSSION

The phases used to quantify progress are coded as in Vallani *et al*., 2002. **Case 1** = on the first visit the dog was in F1/F2; after one month it was in F4, at 45d in F5, at 55d in F6 (all time values are calculated from the beginning of the rehabilitation program). *Follow up*: at two months it was in F6. **Case 2** = on the first visit it was in F1; at two and a half months in F4; at three months in F5 and at three and a half months in F6. *Follow up*: at 5 months it was in F6. **Case 3** = (due to the severity of the symptoms the neurological numerical phases classification was used in this case) on the first visit the dog was in F1; at 30d in F2/3; after 45d in F4; after three months in F6 (i.e. F5 for orthopaedic patients). *Follow up*: at four months the dog was in F6. **Case 4** = on the first visit the dog was in F1, it dragged its feet and rested the dorsal part of the feet on the ground; at 30d it was in F4; at 50d in F5. *Follow up*: at 5 months the dog was in F6.

We conclude that the use of global postural techniques thus appears to be a useful addition to the rehabilitation programs of neurological and orthopaedic canine patients.

ACKNOWLEDGEMENTS

The Authors wish to thank Simona Normando and the colleagues who referred the patients.

REFERENCES

Basaglia, N., 2000. *Trattato di Medicina Riabilitativa* (Gruppo Editoriale Idelson-Gnocchi, Roma)

Buzzi, A.M., Guidi Fabbri, C., 1996. *Le Metodologie Posturali in Funzione Educativa e Rieducativa* (Armando Editore, Roma)

Mountcastle, V.B., 1977. *Trattato di Fisiologia Medica*, (Piccin Editore, Padova)

Ruch, T.C., 1976. Sensazione Somatica. In: T.C. Ruch, H.D. Patton (eds.), *Fisiologia e Biofisica* di J.F. Fulton – W.H. Howell (Società Editrice Universo, Roma)

Souchard, P.E., 1994. *Basi del Metodo di Rieducazione Posturale Globale* (Editore Marrapese, Roma)

Vallani, C., Normando, S., Carcano, C., Piccolo, G., Galardi, V., Longhi, P. and Padovano, R., 2002. Uso di una piattaforma biomeccanica nel programma di riabilitazione di pazienti canini ortopedici e neurologici. *Atti della Società Italiana delle Scienze Veterinarie*, LVI, 327–328

Villeneuve P., 1996. *Pied, Équilibre & Posture*. Editions Frison-Roche, Paris, France