Clinical Proteomics Journal
Copyright © Humana Press Inc.
All rights of any nature whatsoever are reserved.
ISSN 1542-6416/04/01:235–248/$25.00

Original Article

Development of Competitive ELISA for Neosporosis by Employing Immunoproteomics

Yong-seung Shin,[1] Eung-goo Lee,[2] Gee-wook Shin,[1] Young-rim Kim,[1] Eun-young Lee,[1] Jae-hoon Kim,[3] Hwan Jang,[4] Dae-yong Kim,[5] Yong-hwan Kim,[1] Gon-sup Kim,[1] Myung-deuk Suh,[1] and Tae-sung Jung[1,]*

[1]Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju, Republic of Korea; [2]Department of Molecular Parasitology, School of Medicine, Sungkyunkwan University Suwon, Republic of Korea; [3]Department of Veterinary Medicine, Cheju National University, Jeju, Republic of Korea; [4]National Veterinary Research and Quarantine Service, Anyang, Republic of Korea; [5]Department of Veterinary Pathology, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea

Abstract

In this study, proteomics was used to explore the antigenic proteins that are involved in cross-reactivity during serodiagnosis between *Neospora caninum* (*N. caninum*) and *Toxoplasma gondii* (*T. gondii*). Competitive enzyme-linked immunosorbent assay (C-ELISA) developed by proteomics shed a new light on the infection of *N. caninum*. Cross-reactivity of antigenic proteins between *N. caninum* and *T. gondii* tachyzoites was explored by using the conventional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (1-DE) and two-dimensional gel electrophoresis (2-DE) immunoblot. The proteins were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The protein expression patterns in the immunoblot profiles of *N. caninum* were similar to bovine, chicken, and rabbit anti-*N. caninum* serum, but they were not similar to rabbit anti-*T. gondii* serum. Band at 79 kDa, HSP70, and actin on immunoblot profiles

*Author to whom all correspondence and reprint requests should be addressed:
Dr. Tae-sung Jung, Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, 900 Gazwa-Dong, Jinju, Gyeong-nam, 660-701, Republic of Korea.
Tel: +82-55-751-5822, Fax: +82-55-751-5803.
E-mail: jungts@nongae.gsnu.ac.kr

reacted, in general, with bovine, chicken, and rabbit anti-*N. caninum* serum and also with rabbit anti-*T. gondii* serum, respectively. Whereas the band at 144 kDa, and NCDG-1 were detected on bovine, chicken, and rabbit anti-*N. caninum* immunoblot profiles, they were not observed on rabbit anti-*T. gondii* immunoblot profile. These specific antigenic proteins were recorded as species-specific proteins of *N. caninum* against *T. gondii.* Based on the proteome analysis, C-ELISA was developed to screen the cattle infected with *N. caninum* by using *N. caninum* tachyzoite lysate as a coating antigen and chicken anti-*N. caninum* immunoglobulin (Ig)Y as a competitor. C-ELISA was able to detect the antibody of *N. caninum* without cross-reactivity with *T. gondii*. Furthermore, it achieved a fine diagnostic performance in the cases of 162 bovine sera.

Key Words: *Neospora caninum*; *Toxoplasma gondii*; cross-reactivity; competitive ELISA; immunoproteomics.

Introduction

Neospora caninum (*N. caninum*) is an obligate intracellular protozoan parasite belonging to the family Sarcocystidae of the phylum Apicomplexa *(1)*, which resembles *Toxoplasma gondii* (*T. gondii*) *(2)*. This protozoan pathogen was first detected in Norwegian dogs suffering from lameness caused by encephalomyelitis *(3)*. At present, this protozoan has been identified as a serious pathogen for cattle and dogs worldwide *(4)*.

A variety of serological methods such as Indirect Fluorescent Antibody Test (IFAT), enzyme-linked immunosorbent assay (ELISA), immunoblotting, and agglutination test have been tried to find animals infected with *N. caninum (5)*. ELISA has been commonly used to screen a large number of samples thanks to its rapid and large-scale characteristics *(6)*. On the other hands, reducing the cross-reactivity between the family Sarcocystidae, especially *T. gondii* and *Sarcocystis spp*. has been considered an important criterion for developing serological methods for neosporosis *(7,8)*.

Exploration of antigenic proteins was very important for thorough understanding of false positive results because of the cross-reactivity in serological examinations *(9)*. Determinations of antigenic proteins that are competing against each other in cross-reactivity and their use in ELISA have been reported for neosporosis *(10,11)*. Recently, immunoproteomics (two-dimensional gel electrophoresis (2-DE) and its immunoblot studies) was applied in an attempt to reveal antigenic proteins of *N. caninum* by using rabbit anti-*N. caninum* serum, and the number of spots of *N. caninum* antigenic proteins was crossly reacted with rabbit anti-*T. gondii* serum *(12,13)*.

In this study, immunoproteomic analysis was performed to identify specific antigenic proteins of *N. caninum* recognized by antisera against *N. caninum* and *T. gondii*, which provided basic information to avoid cross-reactivity during serodiagnosis between *N. caninum* and *T. gondii*. On the basis of immunoproteomic study, competitive ELISA (C-ELISA) was developed to diagnosis *N. caninum* infection in cattle.

Materials and Methods

If not stated otherwise, all the reagents were purchased from Sigma (St. Louis, USA) and the tissue-culture media from Gibco BRL (Grand Island, USA).

Parasite

N. caninum KBA-2 strain *(14)* and *T. gondii* RH strain tachyzoites were maintained according to Lee et al. *(13)*. Approximately 1×10^8 tachyzoites were purified from the infected Vero cells (GRL 6318; ATCC, Rockville, MO, USA) in a 175 cm^2 flask and stored at -70^oC until use.

Indirect Fluorescent Antibody Test (IFAT)

An IFAT for detection of *N. caninum* and *T. gondii* antibodies was carried out according to Baszler et al. *(15)* and Pappas et al. *(16)*, respectively. A positive result was confirmed by the brightness of the whole tachyzoite body on yellow-green fluorescence. As the *N. caninum* antibodies titer was more than 1:200 and the titers of *T. gondii* was more than 1:100, the host was thought to be infected, respectively. For discussing more accurate, on positive cut-off serum dilutions, the tachyzoite body emitting the normal fluorescence was scored as one-plus (+) positive serum, and if the tachyzoite membrane was thicker because the emitting fluorescence was higher, it was scored as two-plus (++) positive serum.

Tachyzoite Preparation for ELSIA, 1-DE, and 2-DE

Purified tachyzoites were dissolved in 40 m*M* Tris-base, disrupted three times by freeze–thaw cycles in liquid nitrogen, and then sonicated (XL-2020, Misonix Inc. Farmingdale, NY, USA) at 5.5 W for 15 s, eight times on ice slurry. The disrupted tachyzoites were used in three different procedures according to each experiment's purpose. For conventional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (1-DE), 1% (v/v) Triton X-100 and 50% (v/v) 2×SDS Sample Buffer™ were added to suspension of disrupted tachyzoites and boiled for 5 min. For 2-DE, the disrupted tachyzoites were lysed in 2.2 *M* thiourea and 7.7 *M* urea lysis buffer containing 40 m*M* Tris-base, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% w/v dithiothreitol (DTT), 0.5% v/v Triton X-100, and 0.5% v/v IPG-buffer pH 4.0–7.0 (Amersham Bioscience, Uppsala, Sweden), and the lysates were maintained in ice slurry for 1 h. In the case of ELISA, 2% (v/v) Triton X-100 were added to suspension of disrupted tachyzoites. Protein concentrations of all the samples were estimated by the Bradford protein assay *(17)* using bovine serum albumin as a standard.

*Preparation of Anti-*N. caninum *and Anti-*T. gondii *Serum From Animals*

Present study used cow as a major host since she is the major victim of livestock animals. In addition, rabbits and chickens were used as donors of the reagents for developing diagnostic method. Anti-*N. caninum* polyclonal antibodies were raised by immunizing a cow (Holstein, female), rabbits (New Zealand white, 1.5 kg), and chickens (Leghorn, 1 kg). Anti-*T. gondii* serum, moreover, were produced by immunizing rabbits. Prior to inoculation, all the donors were confirmed as *N. caninum* and *T. gondii* negative by IFAT *(15,16)*. To obtain high immune response similar with natural infection, a cow was intravenously inoculated with 1×10^8 live tachyzoites of the KBA-2 isolate suspended in phosphate-buffered saline (PBS). On the other hand, rabbits and chickens were immunized subcutaneously with 1×10^7 sonicated tachyzoites (*N. caninum* KBA-2 strain or *T. gondii* RH strain) mixed with Freund's complete (first immunization) and incomplete adjuvant (second, third, and fourth immunization) at 2-wk intervals, and collected serum or egg yolk when last injection was given 2 wk later. The chicken immunoglobulin (Ig)Y was purified from eggs collected from the immunized chickens using EGGstract® IgY purification system (Promega, Madison, WI, USA). All the animals were cared for in accordance with the guidelines of experimental animal resource of Gyeongsang National University for the care and use of laboratory animals.

1-DE, 2-DE, and Their Immunoblot

1-DE was performed using 10% SDS-polyacrylamide gels (80×100×1 mm) under 10 mA/gel. Isoelectric focusing (IEF) was performed using an IPGphor™ system (Amersham

Bioscience) according to Görg et al. *(18)*, with IPG strips (Immobiline DryStrip™, pH 4.0–7.0, 0.5×3×70 mm; Amersham Bioscience). The prepared tachyzoites samples were mixed with a rehydration buffer (2 *M* thiourea/7 *M* urea, 2% w/v CHAPS, 0.4% w/v DTT, 0.5% IPG buffer, 0.002% w/v bromophenol blue). The tachyzoite samples were focused for a total of 51.1 kVh. After IEF, the IPG strips were subjected to 10% SDS-polyacrylamide gels (80×100×1 mm) and run under same condition of 1-DE. Immunoblot was performed according to Mansfield *(19)*. *N. caninum* tachyzoite lysates separated by 1-DE or 2-DE were transferred onto polyvinyldene fluoride (PVDF) membrane (Immobilon-P™, 0.45 mm; Millipore, Billerica, MA, USA) and incubated with anti-serum at different dilution ratios, such as bovine (1:1), rabbit (1:400), and chicken IgY (1:200). The membrane was treated with 1:2000 dilution of horseradish peroxidase (HRP)-conjugated each being host specific antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). They were soaked in Enhanced Chemiluminescence Solution (ECL™: Amersham Bioscience) for 1 min and exposed to X-ray film (Fuji, Tokyo, Japan).

Protein Visualization, Image Analysis, and Identification

Silver staining of gels, image analysis, in-gel digestion of protein spots on gels, matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) and database searches were performed as Lee et al. *(13)*. For more precise discussing, gel staining, image analysis, and protein identification were performed a minimum of three times and several minor stained bands and spots were not analyzed.

Enzyme-Linked Immunosorbent Assay (ELISA)

Three different types of ELISA—conventional ELISA, C-ELISA, and Herdchek®:Anti-*Neospora* ELISA kit (IDEXX Laboratories, Westbrook, ME)—were compared for their specificity and sensitivity. Basically, antigens coated plates and sera of cow or rabbit, or IgY of chicken as reagents were titrated by the checker-board method and established the amount *(20)*. First, the conventional ELISA was performed according to Osawa et al. *(21)* with slight modifications. The amount of antigens coated plates was set as 200 ng/well. The whole sera for testing were added to the plates, and the HRP-conjugated anti-bovine IgG (1:5000) (Jackson ImmunoResearch Laboratories) were also added. The antigen–antibody complex was determined by using the substrate (3,3′,5,5′-Tetramethylbenzidine dihydrochloride [TMB]). The reaction was stopped by the addition of 2 *M* H_2SO_4 and the optical density (OD) of wells was determined at a wavelength of 450 nm using a microplate reader (Benchmark®, Bio-Rad, Hercules, USA). The cut-off OD value of the conventional ELISA was estimated with 20 bovine negative control sera in IFAT for *N. caninum* and *T. gondii* as Mean OD value + 3 SD.

The C-ELISA was performed according to Baszler et al. *(22)* with modifications. Whole bovine sera were added to two wells coated with *N. caninum* tachyzoites lysate, and chicken anti-*N. caninum* IgY diluted 125-fold was added, serially. HRP-conjugated rabbit anti-chicken IgY diluted 1:5000 (Jackson ImmunoResearch Laboratories) were added to the plates. The amount of bound chicken anti-*N. caninum* IgY was measured by adding TMB solution. The reaction was stopped and measured the OD at 450 nm. The cut-off OD value of C-ELISA was estimated with 20 bovine negative control sera in IFAT for *N. caninum* and *T. gondii* as Mean OD value − 3SD.

Herdchek®:Anti-*Neospora* ELISA was performed according to the manufacturer's procedure. The procedure was basically very similar to the conventional ELISA employed at this experiment. Serum samples with

sample/positive control (S/P) ratio of less than 0.5 were classified as negative for *N. caninum* antibodies, and if the S/P ratio was greater than or equal to 0.5, the samples were classified as positive for *N. caninum* antibodies. The S/P ratio was estimated in the kit, as S/P = (sample OD value − Mean of negative control samples OD value)/(Mean of positive control samples OD value − Mean of negative control samples OD value).

Field Trial Test

Sera of 80 Korean native cattle and 82 Holstein breeds were examined using the three types of ELISA and IFAT to evaluate the efficacy in the field.

Statistical Analysis

Sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), and the degree of agreement of all ELISAs with IFAT and their correlations were calculated using Yates-corrected chi-square test in categorical data analysis by Web based program (http://members.aol.com/johnp71/ctab2x2.html). Statistical significance between the tests was settled at $p < 0.05$, and for determining the degree of agreement, kappa values of $\kappa > 0.8$ were regarded as excellent, kappa values of $0.8 < \kappa < 0.4$ as moderate and good, and kappa values of $\kappa < 0.4$ as poor *(23)*.

Results

IFAT

The antibody titer of rabbit anti-*N. caninum* and anti-*T. gondii* serum showed a rate of 1:1600, respectively, whereas the chicken anti-*N. caninum* IgY and bovine anti-*N. caninum* serum exhibited a rate of 1:800, respectively.

1-DE Immunoblot

1-DE immunoblot profiles for *N. caninum* tachyzoites lysate were able to exhibit 6, 7, and 14 antigen bands with bovine, chicken, and rabbit anti-*N. caninum* serum, respectively (Fig. 1). Of the bands, a number of bands, such as located at 21, 29, 36, 47, 79, and 144 kDa were exhibited commonly on immunoblot profiles of *N. caninum* tachyzoites lysate with each anti-*N. caninum* serum. In the case of immunoblot profile for *N. caninum* tachyzoits lysate with rabbit anti-*T. gondii* serum, four antigenic bands were observed. The bands were also found commonly on immunoblot profiles probed with rabbit anti-*N. caninum* serum except for the band No. 30 (44 kDa), which was only recognized by rabbit anti-*T. gondii* serum used. The bands detected were listed in Table 1.

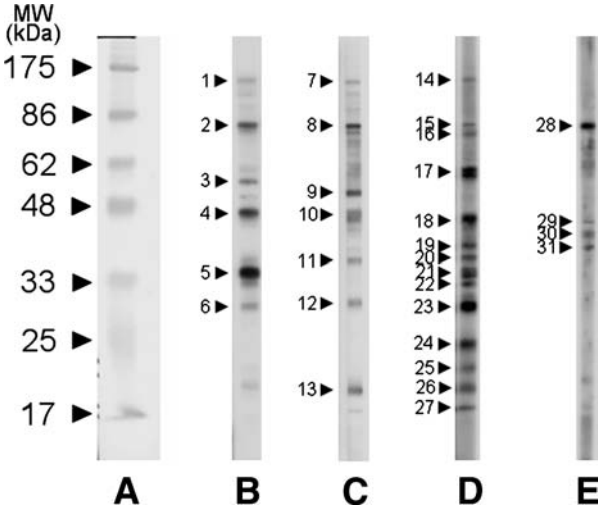

Fig. 1. One-dimensional gel electrophoresis immunoblot profiles of *Neospora caninum* tachyzoites lysate using serum from bovine, chicken, and rabbit anti-*N. caninum* and rabbit anti-*Toxoplasma gondii*. **(A)** Prestained protein marker (New England Biolabs Inc. Beverly, MA, USA). **(B)** Bovine anti-*N. caninum* serum. **(C)** Chicken anti-*N. caninum* IgY. **(D)** Rabbit anti-*N. caninum* serum. **(E)** Rabbit anti-*T. gondii* serum.

*2-DE Immunoblot Profiles With Bovine, Chicken, and Rabbit Anti-*N. caninum *and* T. gondii *Serum*

Very similar 2-DE immunoblot expression patterns were observed for *N. canninum*

Table 1
List of Bands on One-Dimensional Gel Electrophoresis Immunoblot Profiles of *Neoplasma caninum* Tachyzoites

	Anti-*N. caninum* serum			Anti-*Toxoplasma gondii* serum
MW (kDa)	Bovine (lane B)* Band no.	Chicken (lane C) Band no.	Rabbit (lane D) Band no.	Rabbit (lane E) Band no.
144	1	7	14	
79	2	8	15	28
75			16	
61			17	
55	3			
52		9		
47	4	10		
46			18	29
44				30
40			19	31
39			20	
38		11		
36	5		21	
33			22	
30		12		
29	6		23	
26			24	
23			25	
21		13	26	
18			27	

*Lane on Fig. 1.

tachyzoites lysate between bovine anti-*N. caninum* serum (Fig. 2), chicken anti-*N. caninum* IgY (Fig. 3), and rabbit anti-*N. caninum* serum 2-DE immunoblot profile (Fig. 4). But different numbers of antigenic spots were found in the sera used. For example, 15, 28, and 28 spots were observed from the bovine, chicken, and rabbit serum against *N. caninum* tachyzoite proteins, respectively. In this study, five proteins were recognized by MALDI-TOF MS analysis and the common antigenic proteins, which were exhibited on all immunoblot profiles probed with anti-*N. caninum* serum, were HSP70, actin, and NCDG-1. The spots identified with their protein name are described in Table 2, and spots presented on two or more immunoblot profiles, which were remarked as Alphabets, are listed in Table 3.

In contrast, 2-DE immunoblot profile for *N. caninum* that reacted with rabbit anti-*T. gondii* serum (Fig. 5) showed differences from anti-*N. caninum* serum. A total of 15 antigenic spots were detected, and three spots, namely, HSP70, tubulin β-chain, and actin, were identified by peptide mass fingerprinting (PMF) analysis.

Establishment of C-ELISA

In the checker-board titration for evaluation of chicken anti-*N. caninum* IgY in C-ELISA, the

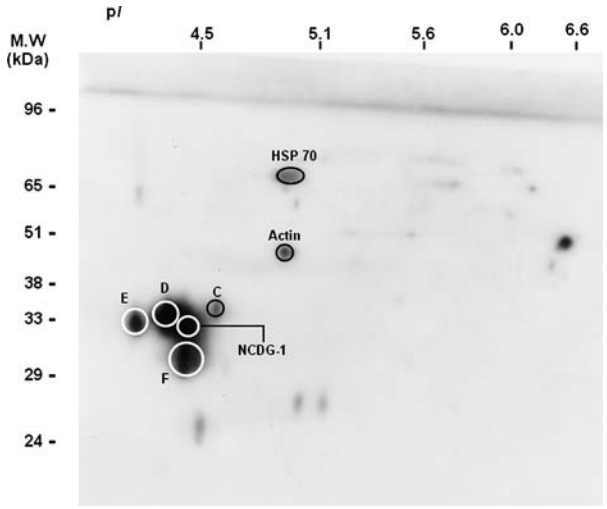

Fig. 2. Two-dimensional gel electrophoresis immunoblot profile of *Neospora caninum* tachyzoites lysate using bovine anti-*N. caninum* serum. A total of 15 antigenic spots were detected and three spots, including HSP70, Actin, and NCDG-1 were identified with peptide mass fingerprinting analysis.

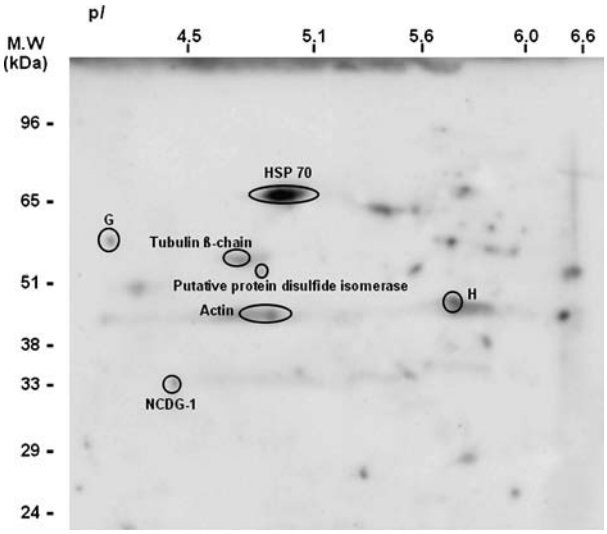

Fig. 3. Two-dimensional gel electrophoresis immunoblot profile of *Neospora caninum* tachyzoites lysate with chicken anti-*N. caninum* IgY. A total of 28 antigenic spots were detected and 5 spots, including HSP70, Tubulin β-chain, Putative protein disulfide isomerase, Actin, and NCDG-1 were identified with matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis.

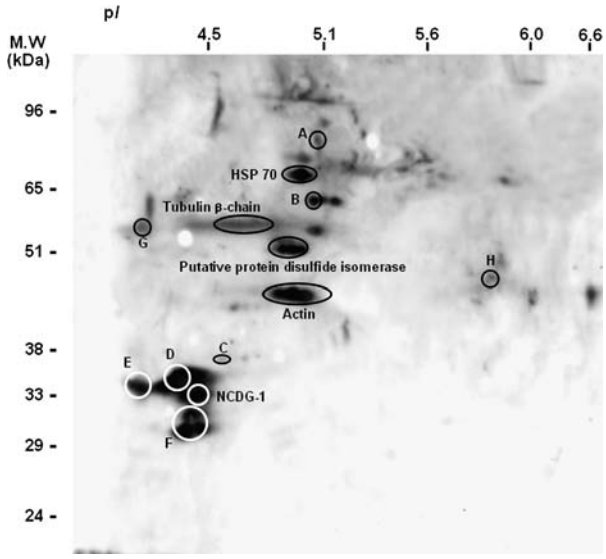

Fig. 4. Two-dimensional gel electrophoresis immunoblot profile of *Neospora caninum* tachyzoites lysate by rabbit anti-*N. caninum* serum. A total of 28 antigenic spots were detected and five spots, namely HSP70, Tubulin β-chain, Putative protein disulfide isomerase, Actin, and NCDG-1 were datamined after peptide mass fingerprinting analysis.

optimal dilution ratio was 1:125 with the whole bovine serum (data not shown). After adding the positive bovine serum diluted 2-fold to the plate coated with *N. caninum* tachyzoites lysate, the chicken anti-*N. caninum* IgY diluted 125-fold was added to the plate to let it compete with the bovine serum. The OD value of chicken IgY slowly increased according to dilution of bovine serum whereas bovine serum exhibited a contrary shape (Fig. 6).

*Cross-Reactivity Performance of C-ELISA With Anti-*T. gondii *Serum*

For evaluating cross-reactivity between *N. caninum* and *T. gondii* on C-ELISA, rabbit anti-*T. gondii* serum and chicken anti-*N. caninum* IgY were added to the plate coated with *N. caninum* tachyzoites lysate. No decrease or increase of OD values on chicken anti-*N. caninum* IgY was observed whereas the rabbit anti-*T. gondii* serum that was used instead of

Table 2
List of Protein Spots Identified on Two-Dimensional Gel Electrophoresis Map of *Neospora caninum* Tachyzoites

Protein name	No. of matched peptides	Sequence of coverage (%)	Theoretical MW[a]/p*I*[b]	MOWSE[c] score	Accession No.[d]	Species
HSP 70	13	22	70,627/5.2	6.52E + 04	11277111M	*Toxoplasma gondii*
Tubulin β-chain	20	32	50,060/4.7	2.03E + 10	135499M	*T. gondii*
Putative protein disulfide isomerase	9	27	52,802/5.1	5.63E + 03	14494995M	*T. gondii*
Actin	19	47	41,908/5.0	2.16E + 10	1703160M	*T. gondii*
NCDG-1	7	33	22,495/4.6	2.91E + 04	3023896	*N. caninum*

[a]Molecular weight (MW; Da).
[b]Isoelectric point (p*I*).
[c]Molecular Weight Search (MOWSE).
[d]No. of the National Center for Biotechnology Information (NCBI) protein sequence database.

Table 3
List of Antigenic Spots Presented on Two or More Immunoblot Profiles

Protein name/ spot no.	Average		Anti-*N. caninum* serum			Anti-*T. gondii* serum
	MW[a]	p*I*[b]	Bovine	Chicken	Rabbit	Rabbit
HSP70	68.9	4.94	○	○	○	○
Tubulin β-chain	54.7	4.74		○	○	○
Putative protein disulfide isomerase	52.9	4.89		○	○	
Actin	45.5	4.93	○	○	○	○
NCDG-1	33.1	4.45	○	○	○	
A	86.6	5.08			○	○
B	59.6	5.02			○	○
C	36.3	4.57	○		○	
D	34.7	4.33	○		○	
E	33.5	4.15	○		○	
F	30.8	4.43	○		○	
G	56.3	4.13		○	○	
H	47.9	5.79		○	○	

[a]Molecular weight (MW; kDa).
[b]Isoelectric point (p*I*).
○, detected antigenic protein.

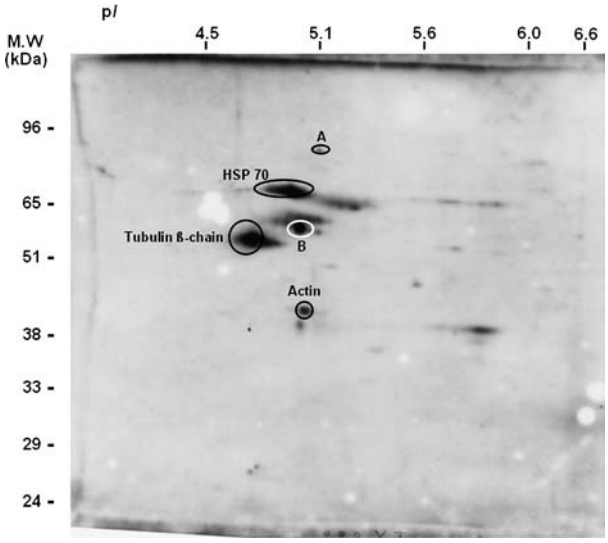

Fig. 5. Two-dimensional gel electrophoresis immunoblot profile of *Neospora caninum* tachyzoites lysate using rabbit anti-*Toxoplasma gondii* serum. A total of 15 antigenic spots were detected and three spots, such as HSP70, Tubulin β-chain, and Actin were identified from database search after matrix-assisted laser desorption/ionization time of flight mass spectrometry.

bovine anti-*T. gondii* showed a decreasing OD value as the rabbit serum got diluted (Fig. 7).

Comparison of IFAT and ELISAs

The efficacy of the C-ELISA developed in this study was examined by comparing the IFAT, the conventional ELISA, and the commercial ELISA (Herd-chek®: Anti *Neospora*) using 162 bovine sera (Table 3).

Sensitivity of C-ELISA was much higher than the other two ELISAs that used (p <0.05), and specificity and PPV of both C-ELISA and Herd-chek® ELISA were higher than the conventional ELISA (p <0.05). However, no significant differences were found in NPV between ELISAs tested (p >0.05). The comparison of degree of agreement and kappa value between ELISAs and IFAT recorded C-ELISA (96.3%, κ = 0.886) and Herd-chek® ELISA (93.8%, κ = 0.802) as an excellent assay, but not the conventional ELISA (87.7%, κ = 0.650).

Discussion

Immunoproteomics was used to explore proteins involved in cross-reactivity between *N. caninum* and *T. gondii*. In this study, we developed a serological diagnostic tool, particularly C-ELISA, to measure *N. caninum* specific antibodies without cross-reactivity with *T. gondii*.

Antigenic bands placed at 79 and 144 kDa of *N. caninum* were commonly recognized by serum raised against the rabbit, chicken, and bovine. Band at 47 kDa detected by chicken and bovine serum, band at 29 and 36 kDa identified by rabbit and bovine serum, and bands at 21 kDa recognized by chicken and rabbit were observed in the *N. caninum* 1-DE immunoblot profiles. These bands have been commonly suspected as antigens detected in various hosts. In other experiments, the bands at 29 and 47 kDa were regarded as the main antigenic bands and suggested to be useful in developing diagnostic markers during serological tests *(24–26)*. But the bands at 21, 36, 79, and 144 kDa presented in this study have not been recognized by other reports, and these bands might be caused by *N. caninum* strain specific, serum collected from different stages of infection, and/or reactivity of antiserum.

In the present study, the rabbit anti-*T. gondii* serum was used instead of bovine anti-*T. gondii* serum, since it was impossible to obtain reliable anti-*T. gondii* serum against cow. As a result, a number of *N. caninum* protein bands (40, 44, 46, and 79 kDa) were recognized by the rabbit anti-*T. gondii* serum. As same this result, a band at 40 kDa was stained by the rabbit anti-*T. gondii* serum against NC-SweB1 and NC-1 *N. caninum* isolates *(27)*. Bands at 43 and 35 kDa were detected by probing the *T. gondii* infected human serum in immunoblot

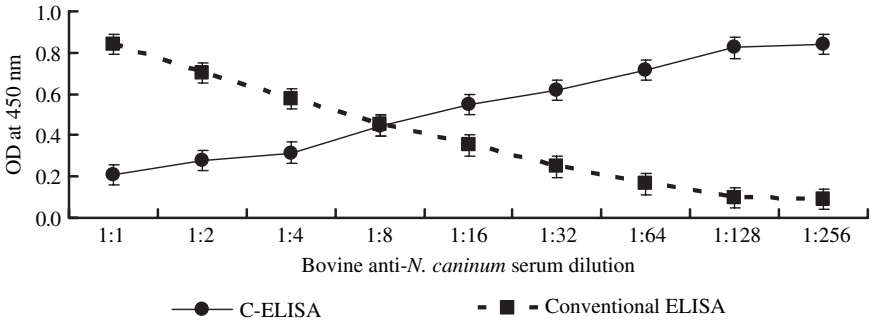

Fig. 6. Evaluation of competitive enzyme-linked immunosorbent assay (C-ELISA) compared with conventional ELISA by the use of bovine anti-*Neospora caninum* serum. The antibodies titers of corresponding *N. caninum* positive control serum were in a steady state of declining whereas the chicken anti-*N. caninum* IgY as a competitor were slowly increasing.

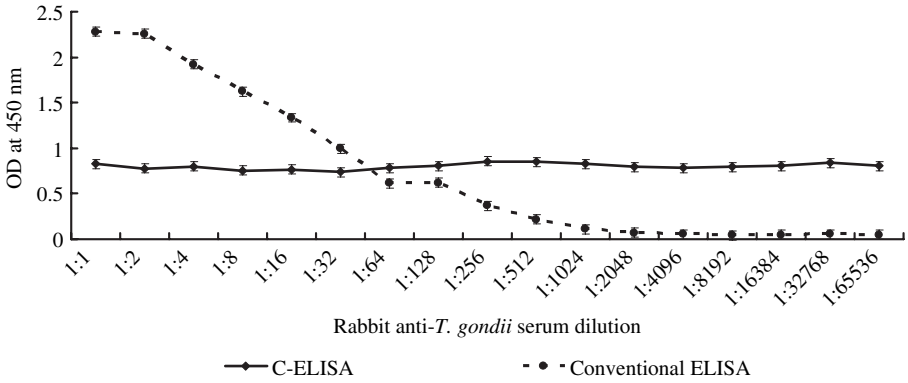

Fig. 7. Evaluation of competitive enzyme-linked immunosorbent assay (C-ELISA) compared with conventional ELISA by the use of rabbit anti-*Toxoplasma gondii* serum. No fluctuation of optimal density (OD) values on chicken anti-*Neospora caninum* IgY was observed whereas the rabbit anti-*T. gondii* serum showed a decreasing OD values as the rabbit serum got diluted.

profile of *N. caninum (28,29)*. These results revealed that there was a distinct cross-reactivity between *N. caninum* and *T. gondii* tachyzoites.

In the 2-DE immunoblot profiles constructed by the bovine, chicken, and rabbit anti-*N. caninum* serum, even though the sera were grown from different hosts, all the images exhibited highly similar antigenic protein patterns. Among the antigenic proteins, HSP70, actin and NCDG-1 as a precursor of host cell invasion and parasite motility *(30–32)* were recognized as common antigenic proteins in all three hosts. On the contrary, the 2-DE immunoblot profiles of *N. caninum* probed with the rabbit anti-*T. gondii* serum were demonstrated less similarities and slightly different locations of main antigens with the bovine, chicken, and rabbit anti-*N. caninum* serum against *N. caninum* tachyzoite. In particular, the rabbit anti-*T. gondii* serum did not recognize the NCDG-1. Similarly, the common antigenic bands at 79 and 144 kDa on 1-DE immunoblot profiles with the bovine, chicken, and rabbit anti-*N. caninum* serum were not found on the immunoblot profile of

Table 4
Comparison of Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value, and Degree of Agreement Between Indirect Fluorescent Antibody Test (IFAT) and Enzyme-Linked Immunosorbent Assays (ELISAs) With 162 Bovine Sera Used

		IFAT									
	Result	2	+	++	Total						
	Total	126	36		162	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Degree of agreement (%)	Kappa value
			10	26							
Conventional ELISA	−	115	9	0	124	75.0[a]	91.3[c,d]	71.1[e,f]	92.7	87.0	0.650
	+	11	1	26	38						
Herdchek® ELISA	−	126	10	0	136	72.2[b]	100.0[c]	100.0[e]	92.6	93.8	0.802
	+	0	0	26	26						
C-ELISA	−	126	6	0	132	83.3[a,b]	100.0[d]	100.0[f]	95.5	93.8	0.886
	+	0	4	26	30						

[a,b,c,d,e,f] Significant differences were found between values with same superscripts at $p < 0.05$ by Yates-corrected chi-test.

the rabbit anti-*T. gondii* serum except a 79 kDa band. In turn, the common spots, such as HSP70 and actin on 2-DE immunoblot profiles and band at 79 kDa on 1-DE immunoblot profile were thought to be antigenic proteins inducing the cross-reactivity between *N. caninum* and *T. gondii*. Whereas NCDG-1 on 2-DE immunoblot profile and bands at 144 kDa on 1-DE immunoblot profile were recognized only by anti-*N. caninum* serum, whose proteins were thought to be species-specific proteins of *N. caninum* compared with *T. gondii*.

Most of the ELISAs have been using crude organism (e.g., tachyzoites lysate) for coating antigens as in the present study. The majority of these coating antigens were probably derived from intracellular or cytoplasmic origin. However, the specificity and sensitivity of intracellular antigens were questionable as *T. gondii* assays had proved *(33)*. To cope with these flaws, surface membrane antigens or serum capable of recognizing specific antigens have been used to increase both specificity and sensitivity during serological tests *(22,34)*. But development of these alternative methods is a time consuming costly work. The present study found that antigens on 1-DE and 2-DE immunoblot profiles of *N. caninum* recognized by chicken anti-*N. caninum* IgY had common antigens founded other immunoblot profiles probed with anti-*N. caninum* serum such as bands at 79 and 144 kDa, HSP70, actin, and NCDG-1. But, they displayed patterns different from the profile probed with the rabbit anti-*T. gondii* serum, particularly NCDG-1. These facts enabled us to implement the chicken anti-*N. caninum* IgY as a fine competitor against bovine antibodies in performing the competitive ELISA for diagnosing *N. caninum* infection. Of course, rabbit

anti-*N. caninum* serum was also seemed to be useful as a competing reagent. But, it was shown little higher SDs of positive/negative results of C-ELISA than that of chicken (data not shown). Moreover, IgY used as competitor for *N. caninum* C-ELISA in this study has various advantages: chicken IgY has high affinity and long persistence in the yolk *(35,36)*, the isolation of IgY is not only a much easier and more reliable procedure but also a method of biological production favorable to animal welfare *(37,38)*; and it is easy to collect a large amount of IgY using a number of immunized chicken eggs *(39)*.

In the process of establishing C-ELISA, the antibodies titers of corresponding *N. caninum* positive control serum were in a steady state of declining whereas the chicken anti-*N. caninum* IgY as a competitor were slowly increasing. In addition, the antibodies of *T. gondii* showed affinity with *N. caninum* antigens coated plate as well as the titer of anti-*T. gondii* was fluctuated, there were no variations of OD value in C-ELISA. These results imply that the C-ELSIA developed with chicken anti-*N. caninum* IgY worked well and made it possible to prevent false positive results of serological test caused by cross-reactivity with *T. gondii* infection. As stated above, however, the implementation of chicken IgY is highly useful, no reports have been published to date on either raising chicken IgY against *T. gondii* and *N. caninum* to employ serological methods or C-ELISA with chicken IgY, or both.

The conventional ELISA did not guarantee prevention of cross-reactivity that led to producing false positive results *(6)*, but the Herd-chek® ELISA was reported to prevent false positive results *(40)*. C-ELISA exhibited a significantly higher sensitivity, specificity, PPV, NPV, and degree of agreement compared to the conventional ELISA. However, no differences were observed except sensitivity when compared with Herd-chek® ELISA. The results of present study indicated the possibility that C-ELISA would not produce false positive results.

Sensitivity of C-ELISA with chicken IgY (83.3%) and Herd-chek® ELISA (73.2%) demonstrated a value lower than the other reports published *(15,21,22,40–42)*. The cause of this relatively low sensitivity in ELISA seems to be related to the sensitivity on IFAT. All the ++ positive sera in IFAT were identified as positive in all 3 kinds of ELISAs. However, the sera that were identified as + positive in IFAT turned out to be either negative or positive in both C-ELISA and commercial ELISA. The differences between ELISA and IFAT were thought to be highly related to the host's low immune responses against *N. caninum*, which are frequently observed in cows with fluctuating or declining antibody levels in such circumstance as in postpartum or post-abortion cows *(43)*, recent seroconverting cows, or calves of the postpartum period *(44)*.

Conventional ELISA has been usually developed without noticing specific reactions between antigen and antibodies. In the present immunoproteomic analysis, chicken anti-*N. caninum* IgY was able to recognize common antigenic proteins of *N. caninum* and *T. gondii,* on the other hand, it was capable of reacting antigens specific for only *N. caninum.* It was the reason why chicken anti-*N. caninum* IgY was useful as a fine competitor for *N. caninum* in C-ELISA without false positive results caused by cross-reactivity with *T. gondii*. In addition, the strategy of this study might be highly useful for developing accurate serological diagnostic methods by combining immunoproteomics, for example C-ELISA, since it is capable of informing us which antigenic proteins are involved in the procedure.

References

1. Dubey JP, Carpenter JL, Speer CA, Topper MJ, Uggla A. Newly recognized fatal protozoan

disease of dogs. J Am Vet Med Assoc 1988; 192:1269–1285.
2. Bjerkås I, Presthus J. Immuno-histochemical and ultrastructural characteristics of a cyst-forming sporozoon associated with encephalomyelitis and myositis in dogs. Apmis 1988; 96:445–454.
3. Bjerkås I, Mohn SF, Presthus J. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. Z Parasitenkd 1984; 70:271–274.
4. Dubey JP. Review of *Neospora caninum* and neosporosis in animals. Korean J Parasitol 2003;41:1–16.
5. Jenkins M, Baszler T, Björkman C, Schares G, Williams D. Diagnosis and seroepidemiology of *Neospora caninum*-associated bovine abortion. Int J Parasitol 2002;32:631–636.
6. Björkman C, Uggla A. Serological diagnosis of *Neospora caninum* infection. Int J Parasitol 1999;29:1497–1507.
7. Dubey JP, Lindsay DS, Adams DS, Gay JM, Baszler TV, Blagburn BL, Thulliez P. Serologic responses of cattle and other animals infected with *Neospora caninum*. Am J Vet Res 1996; 57:329–336.
8. Wouda W, Brinkhof J, van Maanen C, de Gee AL, Moen AR. Serodiagnosis of neosporosis in individual cows and dairy herds: A comparative study of three enzyme-linked immunosorbent assays. Clin Diagn Lab Immunol 1998; 5:711–716.
9. Atkinson R, Harper PA, Reichel MP, Ellis JT. Progress in the serodiagnosis of *Neospora caninum* infections of cattle. Parasitol Today 2000;16:110–114.
10. Howe DK, Tang K, Conrad PA, Sverlow K, Dubey JP, Sibley LD. Sensitive and specific identification of *Neospora caninum* infection of cattle based on detection of serum antibodies to recombinant Ncp29. Clin Diagn Lab Immunol 2002;9:611–615.
11. Ahn HJ, Kim S, Kim DY, Nam HW. ELISA detection of IgG antibody against a recombinant major surface antigen (Nc-p43) fragment of *Neospora caninum* in bovine sera. Korean J Parasitol 2003;41:175–177.
12. Heckeroth A, Tenter A, Hemphill A, Innenes E, Buxton D. Evaluation of cross-reactivity between tachyzoites of *Neospora caninum* and *Toxoplasma gondii* by two-dimensional gel electrophoresis. In: Hemphill A, Gottstein B, ed. A European perspective on *Neospora caninum*, Vol. 30. Int J Parasitol, 2000:877–924.
13. Lee EG, Kim JH, Shin YS, Shin GW, Suh MD, Kim DY, Kim YH, Kim GS, Jung TS. Establishment of a two-dimensional electrophoresis map for *Neospora caninum* tachyzoites by proteomics. Proteomics 2003;3:2339–2350.
14. Kim JH, Sohn HJ, Hwang WS, Hwang EK, Jean YH, Yamane I, Kim DY. In vitro isolation and characterization of bovine *Neospora caninum* in Korea. Vet Parasitol 2000;90:147–154.
15. Baszler TV, Adams S, Vander-Schalie J, Mathison BA, Kostovic M. Validation of a commercially available monoclonal antibody-based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. J Clin Microbiol 2001;39:3851–3857.
16. Pappas MG, Lunde MN, Hajkowski R, McMahon J. Determination of IgM and IgG antibodies to Toxoplasma using the IFA test, ELISA, and Dot-ELISA procedures. Vet Parasitol 1996;20:31–42.
17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
18. Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 2000;21:1037–1053.
19. Mansfield MA. Rapid immunodetection on polyvinylidene fluoride membrane blots without blocking. Anal Biochem 1995;229:140–143.
20. Crowther J. Stages in ELISA. In: Crowther J, ed. ELISA; theory and practice. New Jersey: Humana press, 1995:63–98.
21. Osawa T, Wastling J, Maley S, Buxton D, Innes EA. A multiple antigen ELISA to detect Neospora-specific antibodies in bovine sera, bovine foetal fluids, ovine and caprine sera. Vet Parasitol 1998;79:19–34.
22. Baszler TV, Knowles DP, Dubey JP, Gay JM, Mathison BA, McElwain TF. Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. J Clin Microbiol 1996;34:1423–1428.
23. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977;33:159–174.

24. Barta JR, Dubey JP. Characterization of anti-*Neospora caninum* hyperimmune rabbit serum by western blot analysis and immunoelectron microscopy. Parasitol Res 1992;78:689–694.
25. Bjerkås I, Jenkins MC, Dubey JP. Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. Clin Diagn Lab Immunol 1994;1: 214–221.
26. Harkins D, Clements DN, Maley S, Marks J, Wright S, Esteban I, Innes EA, Buxton D. Western blot analysis of the IgG responses of ruminants infected with *Neospora caninum* and with *Toxoplasma gondii*. J Comp Pathol 1998;119:45–55.
27. Stenlund S, Björkman C, Holmdahl OJ, Kindahl H, Uggla A. Characterization of a Swedish bovine isolate of *Neospora caninum*. Parasitol Res 1997;83:214–219.
28. Nam HW, Kang SW, Choi WY. Antibody reaction of human anti-*Toxoplasma gondii* positive and negative sera with *Neospora caninum* antigens. Korean J Parasitol 1998;36:269–275.
29. Tranas J, Heinzen RA, Weiss LM, McAllister MM. Serological evidence of human infection with the protozoan *Neospora caninum*. Clin Diagn Lab Immunol 1999;6:765–767.
30. Dobrowolski J, Sibley LD. The role of the cytoskeleton in host cell invasion by *Toxoplasma gondii*. Behring Inst Mitt 1997;99:90–96.
31. Augustine PC, Jenkins MC, Dubey JP. Effect of polyclonal antisera developed against dense granule-associated *Neospora caninum* proteins on cell invasion and development in vitro by *N. caninum* tachyzoites. Parasitology 1999;119: 441–445.
32. Dobbin CA, Smith NC, Johnson AM. Heat shock protein 70 is a potential virulence factor in murine toxoplasma infection via immunomodulation of host NF-kappa B and nitric oxide. J Immunol 2002;169:958–965.
33. Hughes HP, Van Knapen F, Atkinson HJ, Balfour AH, Lee DL. A new soluble antigen preparation of *Toxoplasma gondii* and its use in serological diagnosis. Clin Exp Immunol 1982; 49:239–246.
34. Uggla A, Buxton D. Immune responses against Toxoplasma and Sarcocystis infections in ruminants: diagnosis and prospects for vaccination. Rev Sci Tech 1990;9:441–462.
35. Gassmann M, Thommes P, Weiser T, Hubscher U. Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. Faseb J 1990;4:2528–2532.
36. Hatta H, Tsuda K, Akachi S, Kim M, Yamamoto T. Productivity and some properties of egg yolk antibody (IgY) against human rotavirus compared with rabbit IgG. Biosci Biotechnol Biochem 1993;57:450–454.
37. Behn I, Hommel U, Erhard M, Hlinak A, Schade R, Schwarzkopf C, Staak C. Use of polyclonal avian antibodies. In: Schade R, Behn I, Erhard M, Hlinak A, Staak C. eds, Chicken egg yolk antibodies, production and application: IgY-thechnology. Heidelberg: Springer-verlag, 2001: 108–210.
38. Devi CM, Bai MV, Lal AV, Umashankar PR, Krishnan LK. An improved method for isolation of anti-viper venom antibodies from chicken egg yolk. J Biochem Biophys Methods 2002;51:129–138.
39. Zhang WW. The use of gene-specific IgY antibodies for drug target discovery. Drug Discov Today 2003;8:364–371.
40. Paré J, Hietala SK, Thurmond MC. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of Neospora sp. infection in cattle. J Vet Diagn Invest 1995;7: 352–359.
41. Björkman C, Lunden A, Holmdahl J, Barber J, Trees AJ, Uggla A. *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. Parasite Immunol 1994; 16:643–648.
42. Björkman C, Holmdahl OJ, Uggla A. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. Vet Parasitol 1997;68:251–260.
43. Conrad PA, Sverlow K, Anderson M, Rowe J, BonDurant R, Tuter G, Breitmeyer R, Palmer C, Thurmond M, Ardans A, and et al. Detection of serum antibody responses in cattle with natural or experimental Neospora infections. J Vet Diagn Invest 1993;5:572–578.
44. Paré J, Thurmond MC, Hietala SK. *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. J Parasitol 1997;83:82–87.