Use of Polyclonal Avian Antibodies

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Introduction

For many years antibodies have been used for quantitation and detection of a lot of different substances. The specific recognition between antibodies and antigens has led to the development of diverse methods for basic research in the sciences as well as for diagnosis in human and veterinary medicine.

In principle, specific antibodies (immunoglobulins) can be gained by collecting serum from vertebrates immunised with substances (structures) which are antigenic (polyclonal antibodies), or they can be produced by the cell-fusion of antibody-

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producing cells with permanently growing myeloma cell-lines (monoclonal antibodies).

Traditionally, the species chosen for antibody production have been taken from mammals, especially rabbits, but recently there has been a growing use of hens.

Hen IgY is the main serum antibody, but its transfer to the egg resembles the placental or colostral transfer of IgG in mammals. The active transfer of IgY from serum to eggs leads partly to a higher concentration in the yolk than in the serum. After birds have been immunised, polyclonal antibodies can be prepared in various ways without blood-taking (see Chapter 3 and Chapter 4).

The properties of prepared IgY enable it to be used for manifold specific antigen-antibody reactions, so these antibodies can be used not only for diagnostic purposes but also for therapeutic ones. The immunisation of hens instead of rabbits with antigens from mammals can be advantageous, especially in the case of phylogenetically highly conserved antigens. Prepared IgY-antibodies can easily be marked, for instance with enzymes, fluorochromes and biotin, and both marked and unmarked IgY-preparations can be kept for quite a time under suitable conditions (see Chapter 4 and appendix, respectively).

Use of polyclonal avian antibodies as primary and secondary antibodies in immunological reactions

This chapter describes several ways of assaying antibodies and antigens. For modern practical purposes, investigators need methods which are specific, sensitive, reproducible, quantitative and quick. Ideally, the methods should need no costly reagents or equipment. They should be effective in measuring many antigens differing in nature and origin, including polyclonal or monoclonal antibodies.

In Subprotocols 1-5 several classical immunoprecipitation techniques are described.

The indirect marking of antigens using an antigen-specific primary Ab and a species specific labeled secondary Ab is advantageous since the wide range of antigen-specific primary Abs (like mouse monoclonal Abs) can be marked by means of only one species-specific labeled secondary Ab. Therefore, methods

are included in this chapter which describe in detail procedures to conjugate an IgY Ab with enzymes or fluorescent markers (see Subprotocols 6-8).

In Subprotocols 9-17 several ELISA variants are described in detail, which use the IgY-Ab as labeled or non-labeled primary Ab, as a capture Ab or as a labeled or non-labeled secondary Ab. Simple to perform Dot-Blot methods as well as a Western-Blot procedure based on IgY-Abs are recorded in Subprotocols 18-20.

The suitability of IgY Ab for immunohistochemical methods is demonstrated in Subprotocols 21-24. In Subprotocols 25-27 the advantageous application of labeled IgY-Abs is displayed in FACS-analysis. The Subprotocol 28 exemplaryly describes an IgY-based RIA and in Subprotocol 29 a rather complicated procedure is described, which uses IgY for a complement fixation test, although IgY does normally not activate mammalian complement. Procedures are shown in this subprotocol to circumvent this problem. In Subprotocols 30-32 IgY is used for prophylactic and therapeutic purposes in veterinary medicine. The application of specific chicken egg yolk Abs in this field

Table 1. Comparison of the sensitivity of several immunological methods

Method	Detection limit (mg/l)	Application	
		Qualitative	Quantitative
Precipitation			
Mancini	10 - 20	-	++
Ouchterlony	10 - 30	++	-
Immunoelectro-phoresis			
Grabar and Willams	10 - 20	++	-
Rocket	0.2 - 1.0	-	++
Two-dimensional	0.1 - 1.0	++	+
Radioimmunoassay	0.0001 - 0.01	-	++
Enzymimunoassay	0.001 - 0.1	-	++

becomes possible because of the high amount of such Abs obtainable from only a few chickens. The use of specific IgY Abs in therapy and prophylaxis of e.g. diseases of the digestive system of mammals in their early life may be an interesting alternative to the use of antibiotics.

A comparison of the detection limits of the various methods is shown in Table 1.

Subprotocol 1
Single Radial Immunodiffusion (SRID) - 'Mancini'

Basically, immunoprecipitation and agglutination (e.g. Subprotocols 1-5) are techniques which are simple in nature and require only the participation of antigen and antibody to produce a visible immunocomplex (IC). In agglutination the antigen is of corpuscular nature, in immunoprecipitation it is of non-corpuscular nature. In precipitation, ICs are formed either at the interphase between an antigen containing and an antibody containing fluid or in gel at an area of diffusion where the ratio of antigen and antibody amounts lies within the equivalence range. Due to its growing size, the precipitate can neither diffuse nor migrate electrophoretically in gels, and different precipitation techniques are based on this immobilisation.

Specific IgY from hens differs from mammalian antiserum, for instance, in the fact that maximal immunoprecipitations occur in a medium of 1.5 M NaCl (high salt) (Benedict and Yamaga 1976). Precipitation can be further increased by adding 5% (w/v) PEG 6000 to the medium (Ntakarutimana et al. 1992). The extent to which these manipulations should be used depends on the questions posed. In parallel investigations using 1.5 M and the 'normal' concentration of 0.15 M NaCl, Polson et al. (1980) found no basic difference in the results. Hence in routinely using IgY in immunoprecipitation-techniques, one generally has the advantage of being able to use the rich experiences of classical serology with mammalian antibodies in the physiological medium (0.15 M) without having to introduce a second buffer system.

For immunoprecipitations, gels have proven their worth as semi-solid carriers. Use has been made of both purified agar and

agarose. Polson et al. (1980) preferred to use agarose rather than agar, since occasionally white haloes formed round the cup containing the IgY. The authors suspected that these haloes were due to a reaction between agaropectin in the agar on the one hand and traces of lysozym in the not completely purified IgY on the other.

Agar is a mixture of polysaccharides, most of which contain the disaccharide agarobiose. Agarobiose contains many reactive side-groups able to absorb various proteins like fibrinogen, lysozym, thyreoglobulin and ß-lipoproteins. For this reason, agar has been fractionated into agaropectin and agarose. Unlike agar, agarose has no absorbent qualities so it is easier to standardise (Ferencik, 1993).

Antigens are added to wells punched in agarose-gel from where they diffuse radially. They react with antibodies (IgY) distributed evenly in the gel and precipitate round each well as a ring. Within the equivalence range, the width of each ring is proportional to the amount of antigen. Known concentrations of antigens (standard curve) serve as a standard of comparison for the quantification of others.

SRID is used for example for quantifying serum-Ig-isotypes but has other uses too.

SRID is often used when so-called rocket-immunelectrophoresis does not lead to a reliable quantification of the sample, owing to insufficient electropheretic migration of the protein to be determined.

Here is the procedure, as shown by finding the concentration of bovine IgG1 in the serum of cattle:

Materials

Equipment

- gel-punchers, preferably 2 mm in diameter
- a surface to be coated with gel, like
  - glass plates or
  - petri-dishes or
  - microscope-slides or
  - foil-materials

- a temperature controlled waterbath
- a cooker (electrical or gas, for melting the agarose)
- dryer (for drying the gels, e.g. a hair dryer)
- pipettes
- pipette-tips

If it is intended to stain the gel after completion of the test, the carrier glass slides should be coated with melted 0.2% agarose, then baked for 1 h at 100°C in a hot-air steriliser. This is advisable to stop the gel from slipping off the carrier during washing processes.

Reagents, solutions

- agarose (e.g. Serva 11397)
- phosphate-buffered saline (PBS, 0.15M), pH 7.2 - 7.4
- precipitating IgY-anti-bovine IgG1. The dilution for use is to be found by trial and error. The example here is 1% in the gel.
- the standard serum from cattle with known IgG1 content. The example here is 10.05 mg/ml.
- polyethylenglycol 6000 (this additive leads to a better precipitate formation)

Procedure

The Mancini-plates are made as follows:

1. Prepare an agarose solution. For a 1.5% solution, heat 0.75 g agarose in 50 ml PBS to a clear solution. Sodium azide may be added in a concentration of 0.02%.
2. Fill each glass tube with 8 ml, then cool it in a water bath to 56°C.
3. Pre-dilute IgY anti-bovine-IgG1 1:20 (PBS), add 2 ml of the diluted serum to each glass tube and mix well with 8 ml of the fluid gel. The agarose-concentration then comes to 1.2% with an antibody-concentration of 1% in the mixture.

4. Pour the gel onto the levelled microscope-slide to a thickness of about 2-3 mm (10 ml of agarose are enough to coat a surface of 50 cm^2 with 2 mm in thickness).
5. (Optional but advisable) Put the plates into a refrigerator for a while, to render the gel firmer.
6. Punch cups of a predetermined pattern in the solidified gel.
7. Fill the cups with cattle-serum, diluted for instance 1:8 or 1:16, as also with a control serum diluted to the same extent in a volume of 2-5µl (dependent on the cup diameter).
8. Incubate the plates in a moist chamber at room temperature for 48 h.

Evaluation is based on the circular precipitation areas. Formation of the precipitate is complete when the latter is enclosed by a white halo.

Note: The precipitate can be measured more easily if stained. For staining, the plates are immersed in 1% saline with 0.02% NaN_3 for 2 days to remove proteins (with several changes), shortly (1 h) rinsed in distilled water and finally dried. The staining can be done with Coomassie brilliant blue or with other protein-stains like azo-carmine or amido-black according to standard protocols.

Note: The period of preparation for staining can be notably shortened.

1. Cover the Mancini-plates with moist filter-paper, leaving no blisters, and with absorbent material on top of it.
2. Place a plate and a suitable weight on the absorbent material for 30 min. The aim is to squeeze water and remaining soluble proteins out of the agarose layer.
3. Remove the pressing device and immerse the plates in tap water for 1 to 2 h. Repeat step 1/2.
4. Dry the plates covered with filter-paper at room temperature or with a hair-dryer.

Evaluation

The reaction area (precipitate) of the sample is compared to the reaction area of a standard as follows:
mg/ml sample = mg/ml control x area of sample / area of control

Results

One alternative is to take the precipitate's diameter (or diameter squared) as a basis, since this is proportional to the concentration of antigens after full formation of the precipitate which as a rule is reached after 2 days incubation. Under these conditions, a straight line is produced by plotting diameter against concentration (method according to Mancini et al. 1965). Another alternative is to measure the precipitate's diameter already after 6-24 h before full formation (Fahey and McKelvey 1965). In this case the diameter is directly proportional to the logarithm of the concentration of antigens, but the results are less accurate, with a variation coefficient of 10% as compared to about 2% with the Mancini technique. The samples are determined by means of a standard curve. With very good antisera, a detection limit of 1-3 mg/l (of, for instance, IgG or albumin) can be reached (see Table 1).

Troubleshooting

It is recommended to put the gel plate in a refrigerator for about 20 min. In this way the gel becomes more solidified and may not be so easily damaged by preparing the sample cups. Take care to not inundate the cups with inadequate sample volumes.The precipitate can be evaluated only if the agar-solution has been mixed thoroughly with the antibody-solution otherwise irregularly shaped precipitates may occur (see Figure 1). The temperature of the liquid gel before pouring should stay below 58°C, so as not to destroy antibodies. According to our experience, the precipitate evolves better at room temperature than in the fridge, as with other techniques. For ring-precipitation, the plates must be perfectly horizontal; and the punched wells must be far enough from the edge of the gel to avoid edge-phenomena (see Figure1). For further details see Behn 1991.

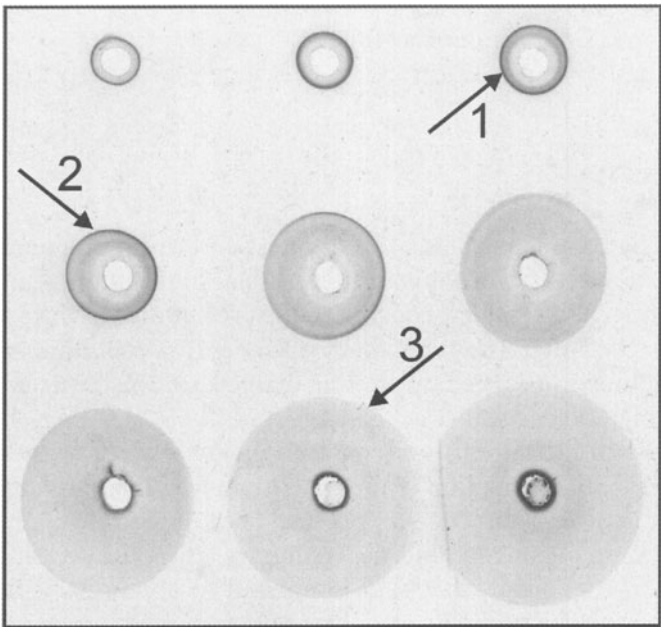


Fig. 1. The quantitative determination of various dilutions (1:2-1:512) of bovine IgG1 by means of radial immuno-precipitation (Mancini-technique). The material used was an IgY anti-bovine IgG1 antibody (1.0%). The developed precipitate was stained with Coomassie brilliant blue G250. 1) The clearly marked ring reveals complete precipitation. 2) Lack of a clear ring reveals irregular precipitation and implies a non-horizontal basis. At low concentrations, this effect is mostly negligible. 3) Here there is incomplete precipitation.

Subprotocol 2
Agar Gel Double Immunodiffusion Test (AGIDT) – 'Ouchterlony Technique'

In AGIDT, antibodies and antigens from neighbouring cups diffuse radially into the gel and in positive cases create line-shaped immune-complexes. The procedure is suitable for showing the presence of either antibodies or antigens. Antibodies are often titred out, but the results are no more than suggestive. The AGIDT's popularity is due to its use in revealing the presence of infections, and the OIE (Office International des Epizooties, 1996) qualifies it in many cases as a prescribed or alternative procedure for the qualitative determination of antibodies.

Materials

Equipment

- Punchers of various kinds. The most widely accepted pattern consists of 6 punch-holes in a circle around a central cup. These holes are normally 4 mm wide and 8 mm apart (other distances are possible). There are many alternative forms.

Otherwise as given under Mancini-technique.

Reagents, solutions

As given under Mancini-technique.

- Equally good results can be obtained by using physiological saline (0.85% NaCl) instead of PBS (see the comments about 'high salt' in the introduction).
- precipitating IgY-anti-CRP
- human CRP-standard serum

Procedure

Production of the AGIDT-plates

1. 1% (w/v) agar in PBS is heated until the solution is completely clear.
2. The hot gel is poured on a glass-slide (or a foil-carrier) in a volume to reach a thickness of about 2-3 mm.
3. After cooling, wells are punched according to a chosen pattern.

Test procedure

1. Fill the wells with antigen suitably diluted and others with corresponding antibody in a way that antigen and antibody containing wells are in neighbouring positions. For titrating an antibody, a central well can be filled with CRP-antigen and the surrounding wells with serially diluted anti CRP-Ab. The volume of the wells should range between 5-20µl, depending on the size of the cup. In the case of Figure 2 the Ab is filled in the central well while surrounding wells have been charged with antigen dilutions.
2. Incubate the materials in the moist chamber for 48 h.
3. Evaluate the results. One or more lines of precipitation are to be regarded as a positive result. In the titration of an anti-

body, the dilution at which a precipitation still visibly occurs under constant conditions counts as the titre.

Results

If the available amounts of antibodies and antigens are small microscopic slides may be used as carrier. A stencil of glass-clear plastic, in which a central reservoir surrounded by further reservoirs, has been cut, is placed on a gel layer about 0.3-0.5 mm thick. The reservoirs are filled with the solutions, with for instance antibodies in the centre and antigens diluted to various extents in the surrounding wells. Through a small hole bored in the base of the reservoir there is a connection to the gel. Usually a blunt needle is pushed through the hole to punch a micro-hole in the gel. The evaluation follows as above.

In qualitative investigations into antigen identities between various antigens in neighbouring cups, note not only the precipitation but also the behaviour of lines of precipitation in the mutual field of reaction. They may create the following patterns.

- Total fusion of both lines in the transition area. This shows that the two antigens are the same (see Figure 2).
- Crossing over without fusion. This shows that the two antigens are different.
- The creation of a spore, if the lines fuse and a spore is created by one of the lines. This shows that the antigens are partly the same.

Troubleshooting

Unfavourable dilution-ratios of antigens and antibodies can yield negative results, so various ratios have to be tried out.

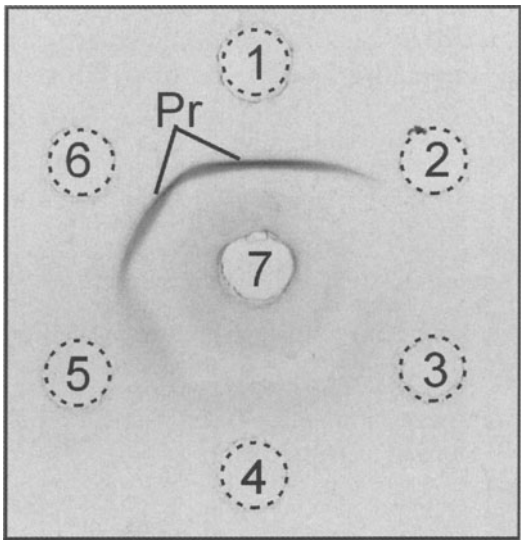


Fig. 2. The qualitative determination of rat CRP by means of the Ouchterlony-technique. To the middle hole (7) are added IgY anti-CRP antibodies, and to holes 1-6 are added various CRP preparations in various dilutions (well 1 contains the serum sample, and wells 2-6 contain isolated CRP in various dilutions). Pr = precipitate.

Subprotocol 3
Immunoelectrophoresis According to Grabar and Williams (1953)

These methods include both electrophoresis and diffusion (Friemel 1991). They are usually carried out in agarose or agar on glass plates or flexible plastic materials. In the first part antigen substances are separated by means of an electric field, and in the second part mono- or polyspecific antibodies diffuse into the gel and form curved precipitates with homologous antigen fractions or the separated protein fractions migrate electrophoretically into an Ab-containing gel. According to the aim, various kinds of agarose with various qualities (like high purity, low electroendoosmosis and low non-specific absorption) can be used. The agarose can be prepared by means of various buffer-systems (like veronal- or tris-barbital, pH 8-9, ionic strength 0.025, containing 0.02% sodium azide or 0.01% merthiolate as preservatives).

This procedure is of only limited use for quantitative work, and for this reason has not become part of routine diagnosis, but it can be used in comparative investigations into antigens and antibodies.

The procedures still common today are:

- classical immunoelectrophoresis according to Grabar and Williams (1953)
- rocket immunoelectrophoresis (Laurell 1966), and
- two-dimensional (crossed) immunoelectrophoresis (Laurell 1965).

These methods have many variations like fused rocket immunoelectrophoresis (see Figure 7, Chapter 4).

Materials

Equipment

- electrophoresis apparatus (Pharmacia LKB, Multiphor II)
- filter-paper (Schleicher Schüll, No. 334497)
- punch cannule (1-3 mm in diameter)
- carrier (for instance microscope-slide)
- levelled plate
- scalpel (to prepare the reservoires for the Ab)

Reagents, solutions

- buffer (for instance a veronal or veronal-sodium buffer, see Chapter 8)
- agarose in electrophoresis buffer (1-2%)
- antigen-dilutions
- Ab-dilutions
- staining-solution (e.g. Coomassie brillant blue, see Chapter 8)

Procedure

There are two stages, electrophoresis and double diffusion.

Electrophoresis

1. Coat the slide with agarose as described.
2. Punch a well in the middle of the carrier and fill it with the antigen to be fractionated electrophoretically.
3. Fill the buffer reservoirs with the appropriate buffer, then use several layers of filter-paper to connect the buffer in the reservoirs with the agarose on the slide.
4. Fractionate the antigen solution by means of an electric current of ca. 5 V/cm for about 60 min.

Double diffusions

1. Cut a trench about 2 mm wide along the slide in the agarose on both sides of the punched hole. The pattern may be changed to a central trench with antigen wells to the left and to the right of the trench.
2. Fill the trench with an antiserum suitably diluted, so that the separated antigens and the antiserum diffuse towards each other. This leaves curved precipitates, which may be stained as described above.

Results

This method is suitable not only for the qualitative demonstration of mixtures of antigens but also for assessing the identities of various proteins (see Figure 3).

Troubleshooting

The concentrations of the antigen and the antiserum must 'match', and the chosen voltage must not be so great as to warm and shrink the gel. To know when the electrophoresis has ended, mix the antigen solution with bromphenol-blue for instance, as this dye is the quickest to migrate and can serve as a marker.

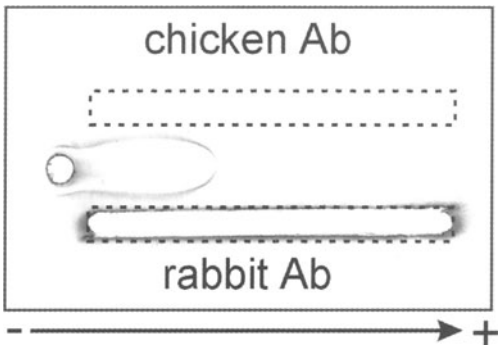


Fig. 3. The qualitative determination of rat CRP by means of immuno-electrophoresis. The material applied is a CRP-preparation (antigen), which has been developed against two different antibodies (monospecific anti-CRP antibodies from hens and rabbits). As both precipitate curves join at the end, both antibodies recognize the same antigen (identical specificity).

Subprotocol 4
Rocket Immunoelectrophoresis (RIE)

This method, also known as electro-immuno-diffusion, was introduced by Laurell in 1966.

Like simple radial immune diffusion according to Mancini, this procedure is based on the diffusion of antigens into an agarose-gel containing antibodies, but the antigens and antibodies migrate respectively to the anode and cathode in an electric field. To move towards the anode, cathodic migrating proteins (e.g. IgG) must be carbamylated. Rocket-shaped more or less pointed precipitates grow from cups filled with antigens. By including standards of known concentration antigens can be quantified.

Materials

See Subprotocol 3.

Procedure

1. Melt agarose to a clear solution, then cool it to about 56°C.
2. Mix the antiserum (which for the quantitative determination of a protein is usually a monospecific antiserum) thoroughly with the agarose. The antibody dilution suitable for this technique must be found by trial and error.
3. Pour the antibody containing gel onto the carrier, cool until the gel has solidified.
4. At a satisfactory distance from the edge of the carrier (about 1 cm, needed for the bridges of filter-paper), punch a row of wells 2 mm wide and 5 mm apart. Provide each plate with sufficient wells for standard samples.
5. Connect the prepared plate with cotton-wicks (filter-paper), as described above, then under slight voltage add the samples to be investigated to the holes.
6. After filling the holes, apply a voltage of 10-15 V/cm. Depending on the electrophoretic properties of the antigen, the rocket develops completely within 30-180 min.
7. Evaluate the plates directly or after staining. The height of the precipitate is directly proportional to the concentration of the antigen and, given the curve of calibrations, can be evaluated simply with the help of an appropriate computer-program.

Results

The RIE (see Figure 4) can also be used the other way round by putting the antigen in the gel, and the antibodies in the well. In this form, the method is sensitive enough to be excellent for identifying serum proteins or other antigens.

Troubleshooting

Too high a voltage can heat the agarose and cause it to shrink. Larger sample numbers should be added to the holes under low

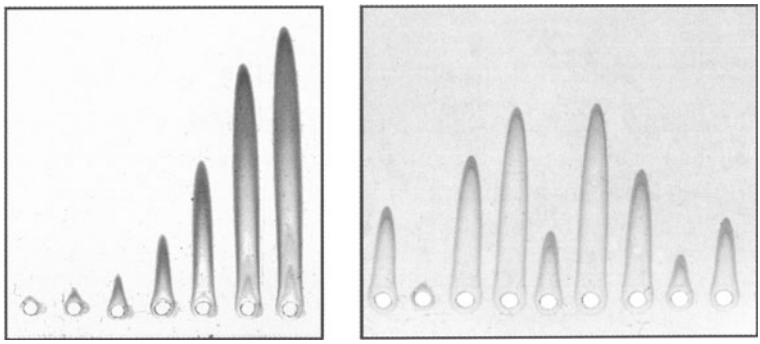


Fig. 4. The quantitative determination of rat CRP by means of rocket immunoelectrophoresis. Use was made of a monospecific IgY anti-rat CRP antibody (1%). The left part of the figure shows an example of a standard series (dilutions 1:1024-1:16), and the right part shows the determination of various samples (rat serum 1:10 diluted).

voltage, otherwise the samples added first may start to diffuse radially and to precipitate. They are then not available for forming the rocket-shaped precipitate, so the results are distorted.

Subprotocol 5
Two-Dimensional (Crossed) Immunoelectrophoresis

The method is a combination of simple electrophoresis and RIE and was developed in the sixties (Ressler 1960, Laurell 1965, Clarke and Freeman 1966).

Materials

Equipment As Subprotocol 4, further, a knife or other suitable means to cut gel strips (containing electrophoretical separated antigen-mixtures) and to transfer them on to glass plates about 10 x 10 cm. Other sizes and materials may be used.

Reagents, solutions As Subprotocol 4.

Procedure

There are two stages, first dimension-electrophoresis and second dimension-crossed electrophoresis.

First dimension-electrophoresis

Carry out a simple electrophoresis on the samples to be investigated in the agarose-gel (see above). Separation is also possible in other gels (PAG) or with other procedures like isoelectric focussing.

Second dimension-crossed electrophoresis

1. After completion of electrophoresis, cut off a strip of gel (2 x 10 cm for instance) containing the separated antigens and transfer it to an empty glass plate.
2. Cover the free part of the glass plate with agarose containing a corresponding antiserum.
3. Carry out a second electrophoresis at right angles to the first (i.e. crossed IE). The conditions are the same as for RIE.

According to their electrophoretic mobility, various precipitates form, where the areas or heights at the completion of electrophoresis are proportional to the concentrations of antigens.

Results

The method is suitable for evaluating mixtures of antigens - like the serum-profile (see Figure 5)- both qualitatively and semi-quantitatively under many conditions. It has variants for identifying antigens in a mixture, like tandem two-dimensional IE, intermediary gel two-dimensional IE and two-dimensional line IE.

Troubleshooting

Pay attention to the relation between the height of voltage and the rise in temperature. The contact between the transferred and the newly poured gel should be as near as possible, with no overlapping. Otherwise as with Subprotocols 1-4.

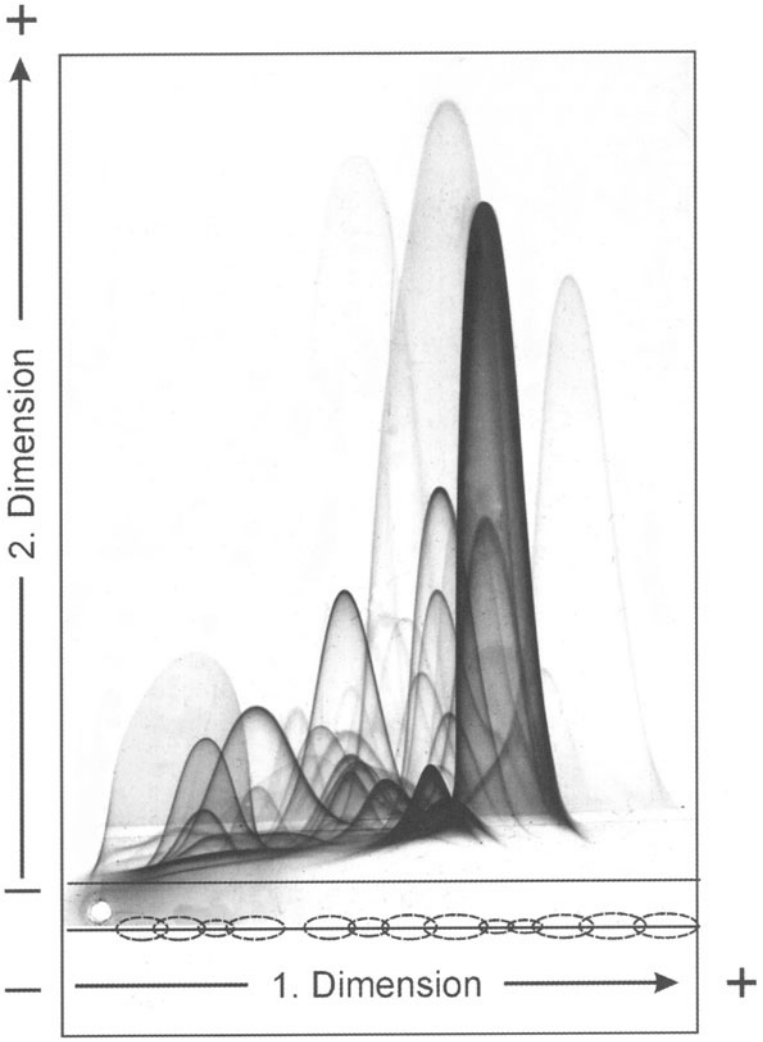


Fig. 5. The protein profile of rat serum (diluted 1:4, 1st dimension). A polyspecific IgY anti-rat serum antibody served as the antibody (4%, 2nd dimension).

Subprotocol 6
Labeling IgY-Antibodies with Biotin

Biotin is a naturally occurring vitamin with a molecular weight of 244 daltons and an extremely strong affinity for avidin and streptavidin. It can easily be coupled to antibodies via an N-hydroxysuccinimide ester without disturbing the antibodies' properties. (Method modified according to Ey et al., 1978)

Materials

Equipment

- magnetic stirrer
- pipettes and tips
- plastic tubes
- dialysis bags
- shaker

Reagents, solutions

- IgY-protein solution (1 mg/ml in PBS or egg buffer, see Chapter 8)
- biotin amidocaproat N-hydroxysuccinimid ester solution (fresh!) (1 mg /100 µl DMSO)
- 3 M glycine
- PBS

Procedure

IgY-antibodies are mixed with biotin solution and cross-linked by incubation.

Note: The biotin solution should be freshly prepared (immediately before the incubation starts).

1. Transfer IgY-protein solution into plastic tube (1 ml)
2. Add biotin solution (25 µl)
3. Incubate 1 h at room temperature while slowly rotating.

4. Stop the reaction and block non-reacted groups for 5 min by adding 80 µl 3 M glycine (rotate slowly).
5. Dialyse the preparation overnight against PBS.
6. Dilute with PBS (1:20) in portions.
7. Store at -20°C.

Results

IgY-biotin conjugates are tested against a panel of proteins to ensure specificity, reactivity and cross-reactivity. The optimal working-dilution in direct and indirect assays should be determined in a titration.

Subprotocol 7
Labeling IgY with Fluorochrome

The conjugation of fluorescein isothiocyanate (FITC) to antibodies is an extremely valuable technique for identifying cell surface molecules by using either fluorescence microscopy or flow cytometry. Fluorescein derivatives are coupled to amino groups of the antibody molecule (IgY). Method modified according to Reisher and Orr (1968, see also Goding 1976, Kronick 1986, and Mohr 1991).

Materials

Equipment

- chromatographic equipment
- photometer
- G25 column (Pharmacia)
- centrifuge

Reagents, solutions

- carbonate buffer pH 9.6 (FITC labeling buffer)
- IgY-protein solution (10 mg/ml) dialysed against carbonate buffer pH 9.6 at 4°C with two or three changes

- PBS
- fluorescein isothiocyanate (FITC, Isomer J, adsorbed to Kieselgur, Serva)
- 5 ml Sephadex G25 (Pharmacia)
- dextran blue

Procedure

1. Mix the dialysed protein solution (100 µl) with 1mg FITC adsorbate.
2. Incubate for 30 min at room temperature while slowly rotating in the dark.
3. Separate the free from bound FITC by means of chromatography on a Sephadex G25 column (with PBS). Dextran blue serves as a marker (due to a mol weight of approx. 2000 kDa dextran blue runs in front of the eluate).
4. Determine and record E_{280} and E_{495}.
5. Determine the working dilution.
   Determine the fluorochrome/protein (F/P) ratio. Mol FITC/protein = $(2.87 \times E_{495}) / (E_{280} - 0.35 \times E_{495})$.
6. Store at -20°C.

Results

IgY-FITC conjugates are tested against a panel of cells to ensure specificity, reactivity and cross-reactivity. F/P ratio should be 3-4 Mol FITC per Mol protein. Other fluorochromes for conjugation are possible, especially rhodamine (TRITC), phycoerythrin and Cy3.

Troubleshooting

The immunoglobulin fraction should be pure, to avoid possible fluorescence due to fluorochrome's binding to accompanying proteins like albumin.

The optimal working dilution in direct and indirect assays should be determined in a titration.

Subprotocol 8
Labeling IgY with Enzyme (e.g. Horse Radish Peroxidase - POD)

Whether used qualitatively or quantitatively, enzyme antibody conjugates are most often used via enzyme-substrate reactions which yield a measurable colour. The variety of enzyme and substrate systems enables conjugates to be used in a lot of different enzyme-linked immunosorbent assays as well as in other tests (e.g. in blot systems or immunohistochemistry). Method according to Nakane and Kawoi 1974).

Materials

Equipment

- photometer (Ultraspec)
- pH-meter
- shaker
- stirrer
- pipettes
- pipette-tips
- dialysis-bag
- centrifuge
- refrigerator

Reagents, solutions

- IgY-protein solution, dialysed against PBS
- PBS
- distilled water
- horse-radish peroxidase (RZ about 3.0)
- 0.1 M sodium m-perjorate solution

- 0.001 M sodium acetate buffer pH 4.4
- 0.1 M sodium borohydride solution
- 0.2 M sodium carbonate solution
- ammonium sulphate solution, cold-saturated with pH 7.4
- 50% (v/v) ammonium sulphate solution (made from cold-saturated ammonium sulphate solution)

Procedure

1. Dilute 5 mg POD with 1 ml distilled water and activate POD with 200 ml 0.1 M sodium m-perjorate solution.
2. Incubate and rotate for 20 min at room temperature.
3. Dialyze overnight at 4°C against 0.001 M sodium acetate buffer pH 4.4.
4. Determine the content of POD photometrically after dialysis by E_{403} in Ultraspec.
5. Mix the activated POD and IgY-sample in a molar relation 1:2.
6. Quickly adjust the pH-value to 9.6 by means of 0.2 M sodium carbonate solution.
7. Incubate and rotate for 2 h in the dark at room temperature.
8. Stop the reaction with 100 ml freshly prepared 0.1 M sodium borohydride solution.
9. Incubate at 4°C for 2 h.
10. Add, drop by drop, cold-saturated ammonium sulphate solution (pH 7.4) in the ratio 1:1 (v/v).
11. Incubate overnight in refrigerator.
12. Centrifuge at 4000 g for 10 min.
13. Wash the sediment thrice with 50% saturated ammonium sulphate solution and centrifuge as above.
14. Dilute the enzyme-antibody conjugate with 4 ml PBS.

15. Dialyse intensively against PBS for 24 h.
16. Determine the molar relation of the POD to IgY by measuring E_{403} and E_{280}.
17. Dilute the enzyme-protein conjugate with PBS/10% foetal calf serum to a concentration of 0.1 mg POD/ml.
18. Store at -20°C in portions or lyophilise.

Troubleshooting

Storage of the preparation at -20°C is recommended instead of lyophilisation.

Comments

Determine the optimal working dilution in direct and indirect assays in a titration. IgY-POD conjugates are tested to ensure specificity and cross-reactivity, and various combinations are suitable as enzymes and substrates for ELISA.

Frequently used marker enzymes and the corresponding substrates

Enzyme	Substrate
(I) horse-radish peroxidase	2,2'-acino-bis(ethylbenzthiacoline-6-sulphonic acid) (ABTS) 405 nm
	o-phenylenediamine (OPD) 492 nm
	3,3',5,5'-tetramethylbenzidine (TMB) 450 nm
(II) alkaline phosphatase	p-nitrophenyl phosphate (pNPP) 405 nm

Subprotocol 9
Direct ELISA to Detect Antigens with a Labeled Antigen-Specific IgY-Antibody

An enzyme-immunoassay (EIA) is any immunoassay in which a substance's concentration is found through the activity of a marker-enzyme. In EIA there are basically two reactions: one immunologic and the other enzymic.

There are many assay-variants, classed roughly according to the reaction-principle:

- non-competitive techniques, with direct or indirect assays (sandwich-assays)
- competitive techniques, with the substance competing with a marked tracer for the same binding site

Only competitive assays rely on a single binding-site.

Moreover, according to the sequence of immunologic and enzymatic reactions, a line can be drawn between homogeneous and heterogeneous assays:

- In a homogeneous assay, both reactions take place in a homogeneous solution.
- In a heterogeneous assay, they take place in different solutions (direct and indirect two-sided assays).

Finally the concept EIA applies to the determination of antigens or haptens, whereas ELISA (enzyme-linked immunosorbent assay) applies to the determination of specific antibodies (see, for instance, Engvall 1980, Tijssen 1985, Porstmann and Porstmann 1991). But in the literature, the terms are used loosely at times. In the following we use the term ELISA.

Outline

Figure 6 shows a schematic view of the assay principle.

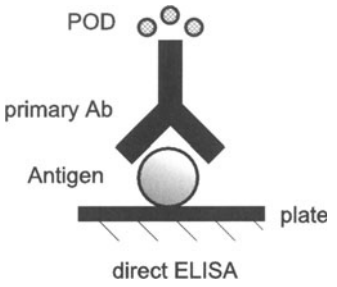


Fig. 6. Scheme for a direct ELISA.

Materials

Equipment

- microtitre-plates (from different suppliers, e.g.Flow Laboratories, Linbro No.76-381-04)
- microtitre-plate reader (e.g. Tecan, Spectra)
- microtitre-plate washer (e.g. Corning, ELISA plate washer)
- multi-channel pipettes (e.g. Bibby Dunn, Labortechnik GmbH)
- pipette tips
- incubator

Reagents, solutions

- coating buffer (carbonate buffer 0.05 M, pH 9.6 or PBS)
- wash buffer, PBS, pH 7.2-7.4/0.05% Tween 20 (Triton-X 100 can be used instead of Tween 20, different wash buffer)
- stop solution, like sulphuric acid (1M)
- blocking solution (to block non-specific binding, several alternatives are possible e.g. 0.5% gelatine, 10% horse serum, 10% gelafusal [Serumwerke Bernburg], 10% haemaccel [Behring-Werke], 1% BSA)
- substrate solution for POD (especially ABTS or TMB or OPD)

- antigen dissolved in coating buffer (1-10 µg/ml, pH 9.6)
- detecting antibody (IgY-POD-labeled) diluted with PBS pH 7.2/0.05% Tween 20

Procedure

1. Coat the plate with 100 µl antigen solution (here mouse IgG) to each well.
2. Incubate overnight at room temperature.
3. Wash the plate with 150 µl PBS/0.05% Tween 20 (wash buffer) thrice.
4. Add 50 µl IgY-antibody enzyme conjugate (POD-labeled hen anti-mouse Ig).
5. Incubate for 1h at 37°C.
6. Wash the plate as above.
7. Add 50 µl (ABTS) substrate solution to each well and leave for 15-45 min at room temperature.
8. Read the plates on a microtitre plate reader, using a 405 nm filter.

Results

This test has been used for determination of the titre of isotype- and subtype-specific antibodies (IgY) against mouse immunoglobulins (Behn et al., 1996).

Troubleshooting

The choice of microtitre plates is important, as the binding-qualities of plates vary a great deal. Before starting an investigation, try to become fully informed or test various makes. Also take so-called edge-phenomena into account. Rows along the edges (horizontal or vertical) may yield results differing from the rest.

The choice of the substrate must depend on the optical filters available for the measuring instrument. OPD is nowadays less commonly used, owing to the suspicion that it is cancerogenic.

Adjust the colour-reaction's period of development to individual requirements. It can range from a few minutes to more than thirty. (For details see Porstmann and Porstmann 1991).

Subprotocol 10
Indirect ELISA to Detect Protein Antigens or Haptens Coupled to a Protein Carrier with an Unlabeled Antigen-Specific IgY-Antibody and a Labeled Secondary Antibody

Outline

Figure 7 shows a schematic view of the assay principle.

Materials

See Subprotocol 9.

Additionally, antibody-enzyme conjugate (e.g. POD-labeled rabbit anti-chicken IgY).

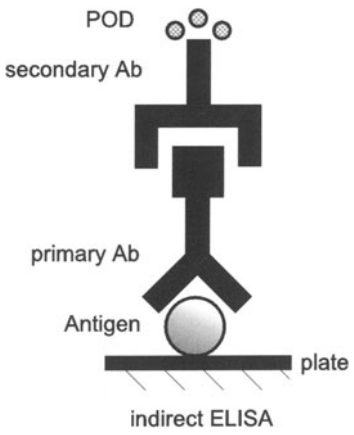


Fig. 7. Scheme for an indirect ELISA.

Procedure

1. Coat the plate with 100 µl antigen in the appropriate dilution (coating buffer) to each well.
2. Incubate overnight at 4°C or for 1 h at 37°C.
3. Wash the plate with 150 µl PBS/0.05% Tween 20 (wash buffer) thrice.
4. Block unspecific binding by incubation with 0.5% gelatine in PBS, 200 µl/well (30 min - 1 h at RT or 37°C).
5. Wash the plate as above.
6. Add 50 µl IgY-antibody preparation (hen anti-x, see Results) suitably diluted to each well and incubate at room temperature or for 1 h at 37°C.
7. Wash the plate as above.
8. Add 50 µl antibody-enzyme conjugate (POD-labeled rabbit anti-chicken IgY) suitably diluted to each well and incubate at room temperature or for 1 h at 37°C.
9. Wash the plate as above.
10. Add 50 µl (ABTS or TMB or OPD) substrate solution to each well.
11. Incubate for 15-45 min at room temperature.
12. Stop the reaction with sulphuric acid (e.g. 1M).
13. Read the plates on the microtitre plate-reader using the corresponding filter (e.g. ABTS-405 nm).

Results

With this assay, various antigens can be determined qualitatively and quantitatively. In the following, only a few references are given. For further references, see (Chapter 8).

- Detection of various isotypes of mouse immunoglobulin by using hen anti-mouse IgM or IgG (Behn et al. 1996).

- Detection of DNP-haptens on bovine gammaglobulin (BGG) as carrier with hen-anti DNP-human serum albumin (HSA) (Behn et al. 1996).
- Detection of p23 (Hlinak et al. 1996).
- Detection of sCD14 (Hlinak et al. 1996).

Troubleshooting

See Subprotocol 9.

Subprotocol 11
Direct Sandwich ELISA for Detecting C-Reactive Protein

Outline

In this assay, the capture-antibody is avian, to avoid misleading reactions with so-called rheumatoid-factors (see Figure 8 for assay principle).

Materials

See above, buffers as described in Subprotocol 9, additionally:

Reagents, solutions

- serum samples from patients with inflammatory disease
- monospecific anti-CRP Ab from hen
- monospecific anti-CRP Ab from sheep (POD-labeled)

Procedure

The procedure is like those already described.

1. Couple the capture-antibody (100 µl antibody-solution/well, purified avian anti-CRP antibody, 2.5 µg/ml) to the plate with carbonate buffer (pH 9.6).

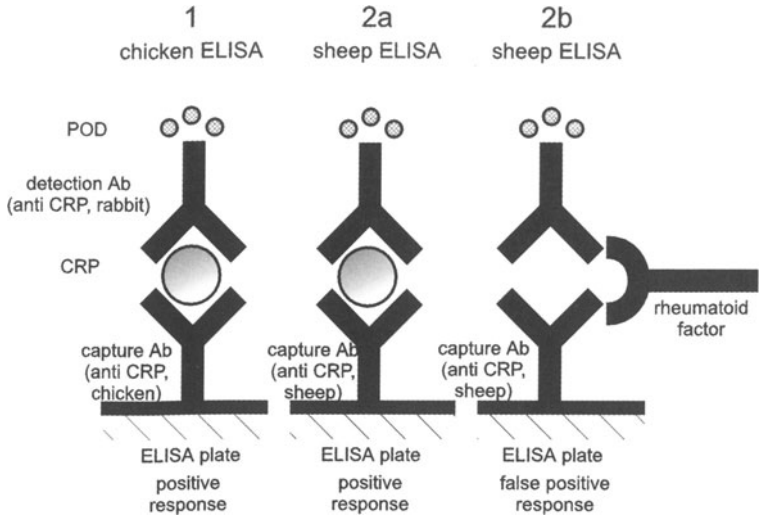


Fig. 8. Direct ELISA for determining CRP. There are two versions of the test: *1.* With hen anti-CRP as capture antibody. *2a, 2b.* With sheep anti-CRP as capture antibody. Due to the binding of rheumatoid factors with immunoglobulins there may be false positive reactions with the second version (*2b*).

2. Incubate for 1 h at 37°C (or at room temperature overnight).
3. Wash the plate with PBS-Triton X 100 (pH 7.4, 0.05%, v/v) thrice with 150 µl.
4. Block unspecific binding by incubation with 0.5% gelatine in PBS, 200 µl/well (1 h at 37°C).
5. Add 50 µl of the serum sample (diluted 1:100 with PBS pH 7.4) and incubate for 1 h at room temperature.
6. Wash as above.
7. Add a POD-labeled anti-human CRP antibody (50 µl, Sigma, diluted 1:1.500).
8. Wash as above.
9. Add 50 µl OPD-substrate solution and incubate at room temperature for 15-45 min.
10. Add 50 µl sulphuric acid (e.g. 1M).
11. Read plates on microtitre plate reader using a 492 nm filter.

Results

The combination of an avian antibody with a mammalian one prevents rheumatoid factors from forming a 'bridge' between capture and primary antibodies (see Figure 8), so results are reliable (Larsson and Sjoquist 1990, Rieger et al. 1996). For details, see Chapter 6.

Troubleshooting

See Subprotocol 9.

Subprotocol 12
Direct Sandwich ELISA for Detecting Bovine IgG2. Use of POD-Labeled Protein G Instead of a Labeled Antibody

This test is suitable for determining the immunoglobulin of mammals. This ELISA functions in this construction only because protein G reacts with immunoglobulin (IgG) of many mammalian species but not with IgY. Protein G also could be used as a binding molecule (instead of a capture antibody) with labeled IgY as the detection-antibody. The assay principle presented here is often less costly than the use of commercial antibodies.

Outline

Figure 9 shows a schematic view of the assay principle.

Materials

See Subprotocol 9, additionally:

- anti-immunoglobulin antibodies from chickens
- immunoglobulin in suitable dilution
- protein G (labeled with POD)
- buffers as above

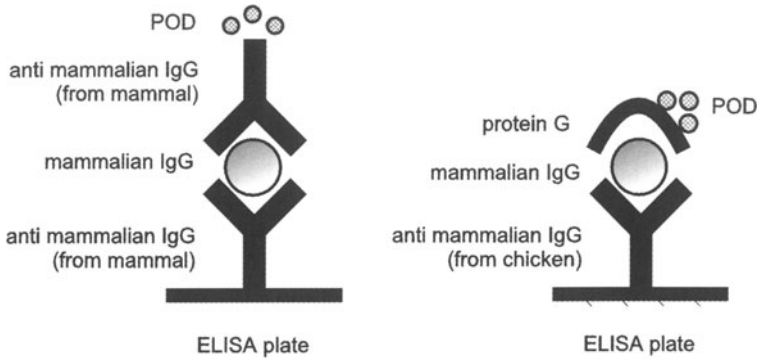


Fig. 9. Direct ELISA for determination of mammalian immunoglobulins (IgG). On the left, the capture antibody is a mammalian anti-IgG antibody, and on the right, an anti-IgG antibody from hens. Through this substitution, the bound IgG can be marked with POD-labeled protein G. The latter has no affinity with hen IgY, so false positive reactions are avoided.

Procedure

1. Coat microtitre plates (Maxisorb, Nunc, Wiesbaden Germany) with affinity-isolated hen anti-bovine IgG2 (100 µl/well, 10 µg Ab/1 ml coating buffer) and incubate overnight at 4°C.
2. Wash the plate with PBS-Tween 20 (pH 7.2, 0.05% Tween 20, v/v) thrice with 150 µl.
3. Block non-specific binding sites with 0.5% gelatine (in PBS, pH 7.2, 200 µl/well), incubate for 1 h at 37°C.
4. Wash as above.
5. Add serum samples from new-born calves or colostrum in suitable dilutions (1:2.000, $\log_2$, 50 µl/well) and incubate for 1 h at 37°C.
6. Wash as above.
7. Add POD-labeled protein G (1:2,500 in PBS-Tween, 50 µl/well) and incubate for 1 h at 37°C.
8. Wash as above.

9. Add substrate solution to each well (TMB, 0.2 mg/ml in 0.1 M acetate-citrate buffer, pH 5 with 0.0005% H_2O_2, 100 µl/well, see Appendix).
10. Stop the reaction after 10 min with 1 M H_2SO_4 (50 µl/well).
11. Read the plates using the corresponding filter (450 nm).

Results

Purified bovine serum IgG2 (0.5 µg/µl, $\log_2$, 50 µl/well) served as standard.

Troubleshooting

See Subprotocol 9.

Subprotocol 13
Sandwich-ELISA With Primary IgY-Antibodies for Detecting *Ascaris suum* - Antigens

Outline

Figure 10 shows a schematic view of the assay principle.

Materials

See Subprotocol 9, in addition:

Reagents, solutions

- capture-antibody (from rabbit) dissolved in coating buffer (6 µg/ml, pH 9.6).
- extract of samples (aqueous extract of faeces from pigs and other species).
- hen anti-SoAg IgY (somatic antigen of *Ascaris suum*) as primary detection antibody (from hyperimmunised hens) in working dilution.

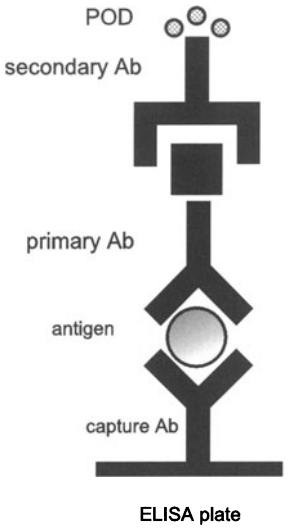


Fig. 10. Scheme for an indirect ELISA with a capture antibody as the antigen binding site.

- commercial POD-labeled rabbit anti-chicken IgY in working dilution.

Procedure

1. Coat the plate with 100 µl capture-Ab solution to each well.
2. Incubate overnight at room temperature.
3. Wash the plate with 150 µl PBS/0.05% Tween 20 (wash buffer) thrice.
4. Block unspecific binding by incubation with 0.5% gelatine in PBS, 200 µl/well (1 h at 37°C).
5. Wash as above.
6. Add 50 µl samples into each well and incubate 1 hr at room temperature.
7. Wash as above.
8. Add 50 µl Ab-preparation in dilution (hen anti SoAg) and incubate 1 hr at room temperature.

9. Wash as above.
10. Add 50 µl POD-labeled rabbit anti-chicken IgY in working dilution.
11. Wash as above.
12. Add 50 µl (OPD) substrate solution to each well and incubate for 15-45 min at room temperature.
13. Stop the reaction with sulphuric acid (e.g. 2.5 N).
14. Read the plates on a microtitre plate reader, using a 492 nm filter.

Results

This test enables infestation with *Ascaris suum* to be shown clearly (note the time lag according to infestation!) not only in the case of pigs but also of humans. ELISA is more reliable than a coproscopic test (Schniering 1995, Schniering 1996).

Troubleshooting

For this test it is crucial to add 50% foetal calf serum to the sample, since the proteolytic activity of the faeces-contents may otherwise destroy the layer of capture-antibodies.

Subprotocol 14
ELISA for Detection of Antibody from Horses Against Dourine (*Trypanosoma equiperdum*)

Outline

The test system is a typical example for commercial ELISA kits used for control of contagious diseases. The O.I.E. (Office International des Epizooties) has accepted this ELISA as an alternative next to the CFT (complement fixation test). Horse sera are

tested in parallel in wells with and without antigen. Differences in extinction between the two are termed "net extinction" (n.Ext.) and a reading ›0.300 is regarded as a positive result.

See Fig. 7 for assay construction.

Materials

Equipment

See Subprotocol 9.

Reagents, solutions

- carbonate buffer (see Subprotocol 9)
- phosphate buffer with Tween 20 (PBS, see Subprotocol 9)
- blocking buffer: carbonate buffer plus 3% fetal calf serum (FCS)
- sample and conjugate buffer: PBS-Tween plus 6% FCS
- substrate indicator system: 40 mg ABTS (see Chapter 8) dissolved in 100 ml citric phosphate buffer (see Chapter 8), just before use 100 µl of 1:40 diluted H_2O_2 is added to 10 ml of ABTS solution
- field and control sera from horses
- antigen: Trypanosomes are isolated from parasitaemic blood from artificially infected rats according to the method of Lanham and Godfrey (1970). 0.5 ml antigen is reconstituted in 5 ml of carbonate buffer, sonicated 3 x 10 sec at 12 microns peak to peak, and centrifuged at 10,000 x g over 4 min. The supernatant is further diluted in carbonate buffer according to results from pretesting.
- conjugate: POD-labeled IgY anti-horse Ig at a pretested working dilution

Procedure

1. Charge wells of columns 2, 4, 6, 8, 10, 12 with 50 µl of antigen, and columns 1, 3, 5, 7, 9, 11 with 50 µl of carbonate buffer.
2. Incubate at 37°C for 40 min in a moist chamber.

3. Wash with tap water and afterwards with carbonate buffer.
4. Add 200 µl per well of blocking buffer and incubate at 37°C for 20 min
5. Wash with tap water followed by 4 wash cycles with PBS-Tween.
6. Add sample serum prediluted 1:100 in sample buffer into 2 neighbouring wells (one with antigen and one without antigen), incubate 30 min at 37°C in a moist chamber.
7. Wash as under 5.
8. Add 50 µl of properly diluted conjugate and incubate as under 6.
9. Wash as under 5.
10. Add 100 µl of ABTS/ H_2O_2 solution to all wells of the plate. The reaction is stopped by the addition of 25 µl/well of a 1:10 diluted household detergent when the positive standard serum diluted to 1:800 reaches a net-extinction of ›0.300.

Results

Results are read by a plate reader at 405 nm and results are given as net extinction. The 1:800 diluted standard serum provides a reaction of 100%. Results from the field sera are expressed in percent to the standard reaction.

Troubleshooting

The system with an integrated control and with the exclusion of non-specific adsorption to the plastic material provides a robust ELISA technique. Misinterpretations can result from reactions caused by contacts with related antigens such like trypanosomes from the T. brucei-group. But these do not occur on the northern hemisphere.

Subprotocol 15
Competitive ELISA

ELISA procedures are notably flexible. Most of the methods now used in routine investigations are non-competitive indirect procedures with an immobilised antigen, but in recent years competitive assays have become more common. Some authors draw a line between competitive and blocking ELISA:

In competitive ELISA, serum samples and detection-antibodies are added at the same time to compete on equal terms for specific binding sites.

In blocking ELISA, antibodies present in the sample compete with labeled detection antibodies for the binding sites. If the former predominate, they block all available sites, so enzyme-reactions are weak or absent.

The use of specific IgY (anti-HSP 70) in a competitive ELISA system is illustrated below in the quantitative detection of heat shock protein (HSP 70) (Gutierrez and Guerriero 1991). The idea is that in the pre-incubation of HSP 70 with a standardised concentration of IgY-anti-HSP 70, the latter bind to HSP in proportion to their concentration, so the results in the following ELISA depend on the amount of bound or non-bound anti-HSP 70 IgY (see Figure 11).

Outline

Figure 11 shows a schematic view of the assay principle.

Materials

See Subprotocol 9, additionally:

- HSP 70 in suitable dilutions
- chicken anti-HSP 70
- buffers as above

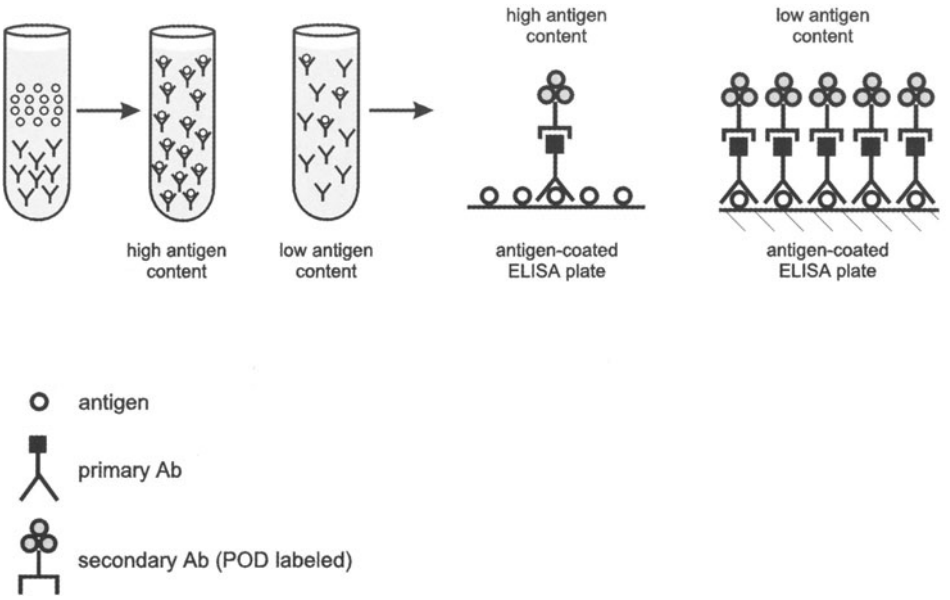


Fig. 11. Scheme for a competitive ELISA. What is shown tallies with stage II (preincubation) and stage III (sample transfer) in Subprotocol 15.

Procedure

ELISA plates

Preparation of the ELISA plates:

1. Coat the plate with HSP 70 as the antigen (100 µl antigen solution in coupling buffer).
2. Wash the plate thrice with PBS Tween 20, 0.05%, pH 7.2)
3. Block the plate with gelatine (Tween 20-PBS, 0.1% gelatine).
4. Wash as above.

HSP 70-standard and samples

Preparation and preincubation of the HSP 70- standard and the samples:

1. Prepare a HSP 70 stock-solution in a concentration of 800 ng/100 µl.
2. Prepare standard-dilutions as follows: ng/100 µl = 1:400, 1:200, 1:100, 1:50, 1:25, 1:12.5.

3. Add equal volumes of the pre-tested anti-HSP IgY and incubate the mixtures.

Transfer of the incubated samples onto the coated ELISA-plates: **Transfer**

1. Transfer the 6 variously diluted standard and assay-samples, pre-incubated anti-HSP IgY, onto the ELISA plate and incubate at 37°C for 2 h.
2. Wash as above.
3. Add AP(alkaline phosphatase)-labeled rabbit anti-IgY conjugate in working dilution, then incubate them at room temperature for 1 h.
4. Wash as above.
5. Add substrate (p-nitrophenyl phosphate, pNPP) (see Chapter 8).
6. Determine the net-extinction (405 nm) through photometry, then determine the HSP-concentration of the sample by comparing it with the standard set.

Results

See Figure 11.

In the sample there was a lot of HSP: anti-HSP IgY was wholly bound at stage II, so was unable to react at stage III.

In the sample there was little or no HSP: anti-HSP IgY was hardly or not bound at stage II, so was able to react at stage III.

Competitive ELISA can be used on samples to detect antigens as well as antibodies.

Troubleshooting

As with other ELISAs.

Subprotocol 16
ELISA to Determine the Content of Mouse Monoclonal Antibodies by Means of a Mouse Ig-Specific Capture-Antibody and a Labeled Detecting Antibody from Chicken (Sandwich)

Outline

For principle see Figure 8.

Materials

See Subprotocol 9, in addition:

- commercial rabbit anti-mouse Ig (capture antibody solution 1-10 mg/ml PBS)
- monoclonal antibodies of various isotypes (the mAb in this system are the antigens to be detected)
- chicken antibody-enzyme conjugate

Procedure

1. Coat the plate with 100 µl capture Ab solution into each well and incubate overnight at room temperature.
2. Wash the plate with 150 µl PBS/0.05% Tween 20 (wash buffer) thrice.
3. Add 50 µl solution containing mouse monoclonal antibodies to each well and incubate 1 hr at room temperature.
4. Wash as above.
5. Add 50 µl POD-labeled chicken anti-mouse IgY in appropriate dilution to each well and incubate at room temperature.
6. Wash as above.
7. Add 50 µl ABTS substrate solution to each well and incubate for 15-45 min at room temperature.

8. Read the plates on the microtitre plate reader using a 405 nm filter.

Results

This assay was successfully used for estimation of content of IgM or IgG in hybridoma cell culture supernatants.

Troubleshooting

See Subprotocol 9.

Subprotocol 17
Indirect ELISA to Detect Antigens With an Unlabeled Primary Antibody and Labeled Secondary Antibody (IgY)

Outline

See Figure 7.

Materials

See Subprotocol 9, in addition:

- antigen solution (1-10 mg/ml PBS)
- unlabeled mouse monoclonal antibody in appropriate dilution.
- POD-labeled chicken anti-mouse immunoglobulin in appropriate dilution.

Procedure

1. Coat the plate with 100 µl antigen solution to be identified to each well and incubate overnight at room temperature.

2. Wash the plate with 150 µl PBS/0.05% Tween 20 as a wash buffer thrice.
3. Add 50 µl unlabeled mAb of different supernatants of hybridoma cell cultures to the wells and incubate for 1 h at room temperature.
4. Wash as above.
5. Add 50 µl POD-labeled chicken anti-mouse immunoglobulin Ab to each well and incubate for 1 h at room temperature.
6. Wash as above.
7. Add 50 µl ABTS substrate solution to each well.
8. Read the plate using the corresponding filter (405 nm).

Troubleshooting

See Subprotocol 9.

Applications

This procedure was successfully used for estimation of antibody production of hybridoma cell cultures (screening).

Subprotocol 18
Proof of Specificity of Avian Antibodies Used Against CCK (Dot Blot)

The blot assay is a simple way of testing the specificity of antibodies. In this protocol various CCK-derivatives are transferred to foil and developed with anti-CCK Abs of different specificity.

Outline

See Figure 12.

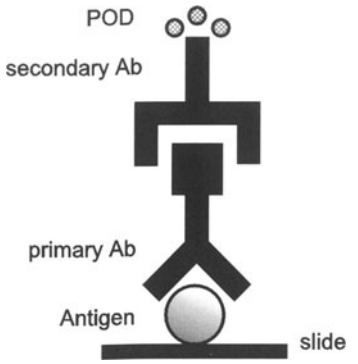


Fig. 12. Scheme for an indirect immunohistochemical marking of an antigen.

Materials

Equipment

- shaker
- pipettes
- pipette-tips
- incubator
- plastic dish

Reagents, solutions

- nitrocellulose sheet like NC 2, pore-size 0.2 mm, Serva
- blot buffer (see Chapter 8)
- Triton X 100
- antigen solution (several CCK-derivatives, see Figure 13)
- paraformaldehyde
- skimmed milk-PBS solution (Glücksklee, skimmed milk powder, Nestlé)
- primary IgY-antibody (anti-CCK Ab from hen) in an appropriate dilution (blot buffer)
- secondary anti-IgY antibody, POD-labeled and diluted in a blot buffer

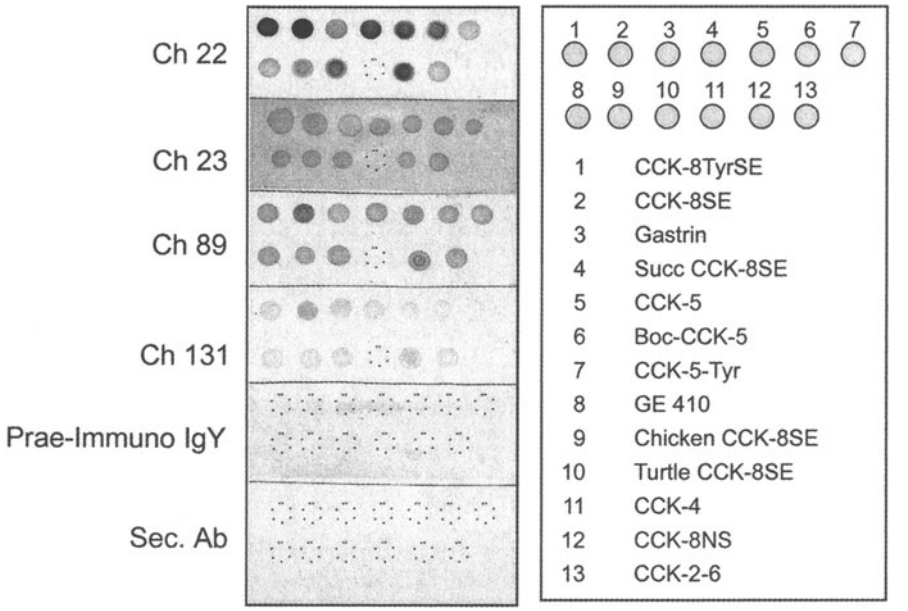
- 15 mg DAB (Sigma) in 50 ml PBS with 1500 ml ammonium nickel sulphate (3% w/v) and with 15 ml H_2O_2 (1 ml 30% H_2O_2 in 30 ml PBS)
- distilled water

Procedure

1. Prepare the nitrocellulose strips, handling them carefully (don't touch them).
2. Wash the sheet in a blot buffer (PBS-Triton X 100) for 10 min and shake slowly.
3. Dry the sheet for about 15 min at 37°C in an incubator.
4. Apply the antigen solution in a dilution range, with 1.5 µl solution per dot, to the sheet through a suitable pipette tip.
5. Dry the sheet for 10 min at 37°C.
6. Keep the sheet for 1 h in a paraformaldehyde atmosphere at 80°C in case peptides are detected.
7. Repeat step 2.
8. Block the sheet in a 5% (w/v) skimmed milk PBS solution and shake slowly (1 h).
9. Repeat step 2.
10. Incubate the sheet in the primary IgY-antibody for 1 h.
11. Repeat step 2.
12. Incubate the sheet in the secondary anti-IgY Ab, labeled with POD, for 30 min.
13. Repeat step 2.
14. Stain the sheet for about 10 min or less in a DAB solution.
15. Stop the reaction by means of distilled water.
16. Dry the sheet.

Results

This is a quick and simple test of the binding of an antibody with a lot of different antigens. Even with protein-antigens, steaming with paraformaldehyde can improve the results (see Figure 13).



Ch 22	Asp-Tyr(SE)-Met-***Gly-Trp-Met-Asp-Phe*** -NH_2-$(Lsy)_4$-PEG-Resine
Ch 23	KLH-$(Gly)_4$-Asp-Tyr(SE)-Met-***Gly-Trp-Met-Asp-Phe*** - NH_2
Ch 89	BSA-Suc-Asp-Tyr(SE)-Met-***Gly-Trp-Met-Asp-Phe*** -NH_2
Ch 131	BSA-Suc-Asp-Tyr(SE)-Met-***Gly-Trp-Met-Asp-Phe*** -Tyr(SE)-NH_2

Fig. 13. Comparison of the specificity of four chicken antibodies, raised against four CCK-derivatives; specificity testings of various IgY antibodies by means of dot-blot methods (indirect detection). The lower list gives information about the structure of the antigens used for immunisation. The antibodies were used in dilutions of 1:200 - 1:400. The various CCK-derivates (equimolar concentrations) were applied in amounts of 1.5 µl. Dependent on the specificity of the Abs used the various derivatives are marked with various intensities, and antibodies Ch22 and Ch89 distinguish between CCK and gastrin. (CCK and gastrin belong to a peptide family, and have the last 5 amino acids [in italic] in common). SE = sulphated, NS = not sulphated.

Troubleshooting

Handle the nitrocellulose sheet carefully, without touching it. The sheet must be wholly dry, or the dots will be irregularly shaped. A critical step is the blocking of non-specific binding. We have good experience with low-fat milk powder.

Subprotocol 19
Dot Blotting, Detection of Rubella-Virus Proteins

Outline

See Subprotocol 18 and Figure 12.

Materials

Equipment

See Subprotocol 18, additionally:

- scanner

Reagents, solutions

- rubella-antigen solution (110 virus units/ml)
- nitrocellulose (BIOMETRA) GM 045
- PROMEGA-Kit A (special buffer concentrate, concentrate: distilled water, 1:1)
- wash buffer I (PBS, 3% skimmed milk powder, 0.2% Tween 20)
- wash buffer II (wash buffer I:solution A, 4:1)
- wash buffer III (solution A:wash buffer I [without Tween 20], 1:1
- substrate buffer (50 ml III + 10 ng DAB + 8 ml H_2O_2, 30%)
- 1 M sodium acetate buffer
- monoclonal anti-rubella antibody (primary mouse antibody B1, IgG2a, diluted in wash buffer I)
- POD-labeled detection antibody (secondary chicken anti-mouse IgG antibody) in appropriate dilution (wash buffer I)

Procedure

1. Prepare the nitrocellulose sheet, handling it carefully (see Subprotocol 18).
2. Apply the antigen solution in a working dilution, with for instance 1 µl solution per dot, to the sheet by means of a pipette with a suitably sized tip.
3. Dry the sheet for 10 min.
4. Wash the sheet for 20 min in blot buffer I and shake it.
5. Incubate the sheet in the primary antibody for 18 h (1:500).
6. Wash the sheet four times in wash buffer I for 15 min.
7. Incubate the sheet in the POD-labeled secondary antibody for 1 h.
8. Wash the sheet four times in wash buffer I.
9. Wash the sheet thrice in wash buffer II.
10. Incubate the sheet once in wash buffer III.
11. Incubate the sheet once in substrate buffer for 15 min.
12. Stop the reaction with 1M sodium acetate buffer for 5 min.
13. Dry the sheet.
14. Evaluate the results by means of a scanner.

Results

See Figure 14.

Troubleshooting

See Subprotocol 18.

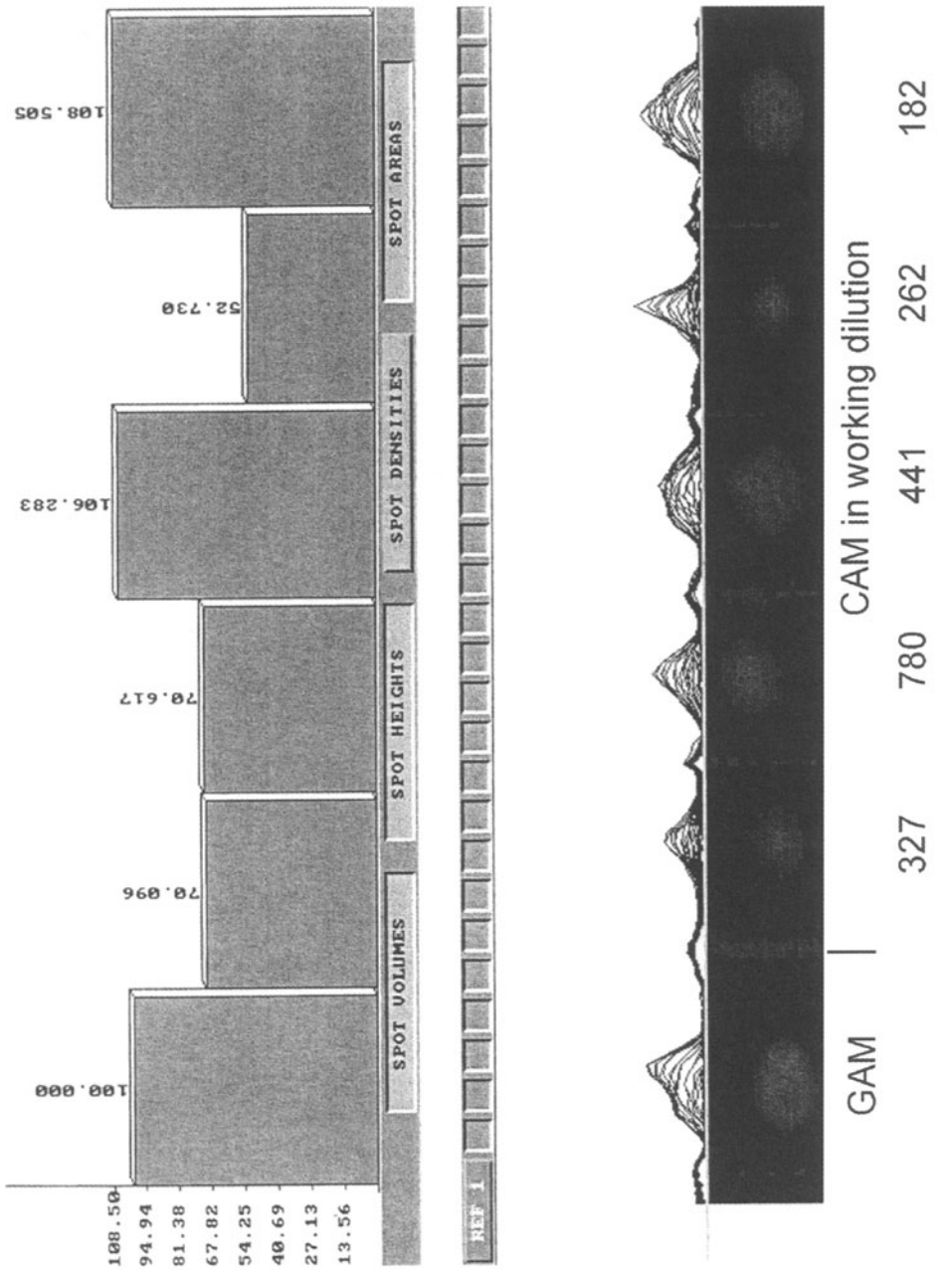


Fig. 14. Dot blot method. Demonstration of Rubella virus protein by means of mouse monoclonal antibody. Shown is a comparison between the efficacy of a commercial POD-labeled goat anti-mouse IgG Ab (GAM) and different POD-labeled chicken anti-mouse IgG Ab (CAM). The lower part of the Fig. shows the original spots and the corresponding scans which are expressed in the upper part as a relative value.

Subprotocol 20
Immunoblotting, Western Blotting

This method is used frequently to demonstrate a specific agent out of a mixture of several agents or to demonstrate the specificity of an antibody.

Outline

The principle of the method is based on an electrophoretical separation of a fluid followed by an electrophoretical transfer of the separated fractions on a suited foil. This foil is further treated as described in Subprotocol 18 (dot-blot).

Materials

Equipment

- polyvinylide-difluoride membrane (Immobilon P, Millipore, pore size 0.45 μm)
- electrophoresis chamber (Protean II xi, BIORAD)
- blotting chamber (Trans-Blot-Cell, BIORAD)
- shaker

Reagents, solutions

- acrylamide/bisacrylamide stock solution, monomer concentration 30%, cross-link concentration 1% (SERVA, Heidelberg)
- buffer stock-solution (Tris 77.43 g, sodium acetate 43.83 g, EDTA 9.37 g, in 1000 ml dist. water)
- electrophoresis buffer (buffer stock-solution 75 ml, SDS 1.2 g, in 1125 ml dist. water)
- sample buffer (32.5 ml dist. water, buffer stock-solution 3.0 ml, SDS 7.5 ml, 2-β-mercaptoethanol 2.5 ml)
- LPS solution of different origin
- Mol weight marker (Rainbow, Amersham)
- bromphenol blue (marker for electrophoresis)

- methanol
- blot-buffer (25 mM Tris, 192 mM Glycin, 10% methanol)
- PBS-Tween 20 [0.1%]
- PBS-Tween 20 (0.1% + 3% skimmed milk, Glücksklee, Nestlé)
- chicken anti-LPS Ab diluted 1:100 in PBS-Tween
- POD-labeled anti-chicken IgY diluted 1:5,000 (SIGMA)
- substrate solution (DAB, see Subprotocol 18 or Chapter 8)

Procedure

Step 1 Separation of LPS-samples using a modified continuous system according to Laemmli 1970

1. Prepare the separation gel (gradient 12-16%, pH 7.4, SDS 1%, stacking gel 4% otherwise as with the separation gel), gel size 140 x 160 x 1 mm.
2. Dilute LPS stock solutions (10 mg/ml) with sample buffer (1:9, vol/vol) and incubate for 5 min at 100°C.
3. Load the LPS sample and the mol weight marker, respectively on the gel in a volume of 20 µl/slot.
4. Run the electrophoresis (40 V, 30 mA/gel, ca. 18 h), under these conditions the bromphenol blue line migrates approximately 10 cm.
5. Keep the temperature during the electrophoresis at 5°C (flow cooler) and exchange continuously the buffer between the upper and lower buffer reservoire (peristaltic pump).

Step 2 Transfer of the separated substances on the blot membrane modified according to Towbin et al. 1979, and Towbin and Gordon 1984

1. Treat the blot membrane with methanol for 2 min and wash subsequently with distilled water.

2. Prepare the blot-sandwich according to the instructions of the manufacturer.
3. Run the transfer at RT for 18 h (0.1 mA, 10V).
4. After the transfer rinse the membrane shortly in methanol and dry the membrane for 30 min at RT.
5. For orientation stain the mol weight marker with amido black.
6. Block non-specific binding by incubation in PBS-Tween with 3% skimmed milk for 6 h at RT (shake continuously).
7. Wash thrice with PBS-Tween for 15 min.
8. Incubate in the chicken anti-LPS Ab for 18 h at RT (shake continuously).
9. Wash as above.
10. Incubate in the anti-chicken IgY Ab for 1 h (shake continuously).
11. Wash as above.
12. Stain the membrane with DAB as described in Subprotocol 18 and block the reaction with distilled water.
13. Dry the membrane at RT.

Results

See Figure 15.

Troubleshooting

The transfer sandwich must be prepared carefully to avoid air bubbles which can disturb the correct transfer of the substances separated. Otherwise as with Subprotocol 18 (for further technical details see Kyhse-Anderson 1984, Bjerrum and Schaffner-Nielsen 1986).

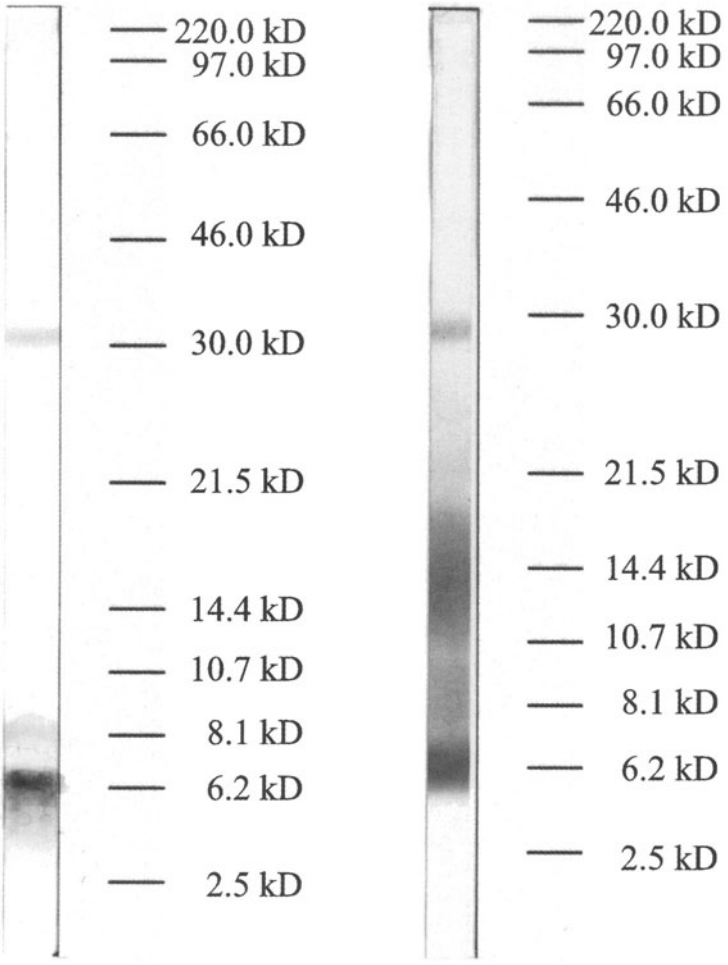


Fig. 15. Western blot. Specificity analysis of chicken antibodies raised against lipopolysaccharide (LPS)-species of different origin. *kD* = kilo Dalton.

Subprotocol 21
Immunohistochemistry, Visualisation of CCK-8 by an Indirect Staining Method

Immunohistochemical processes are used to reveal tissue-structures. In principle, the assays are designed like an ELISA, so there are both direct and indirect forms. Moreover there are various methods, like the biotin-avidin one, for strengthening the specific signal. For the sake of good results, the tissue to be examined should be suitably prepared, for instance by fixing if necessary. Various procedures are common and may lead to varying results (see Cuello 1983, Ambrosius and Luppa 1987).

Outline

See Figure 12.

Materials

Equipment for step 1

- beakers
- water-bath
- filtering apparatus

(The solutions should be filtered to leave no foreign bodies visible on the sections.)

Reagents, solutions for step 1

- picric acid
- formaldehyde
- mercuric chloride
- glacial acid
- distilled water

Equipment for step 2

- 2 peristaltic pumps (e.g. Ismatec MC 360), for a buffer-solution and Bouin's solution respectively
- pressure-bottles
- connecting-tubes
- valves
- scissors and pincettes
- clamp
- button cannula

Reagents, solutions for step 2

- 400 mg/ml cloralhydrate (Merck)
- 200 ml haemaccel/PBS-solution (1:5 v/v)
- 500 ml Bouin's solution

Equipment for step 3

- vibratome, cryo-microtome
- vials, 5-10 ml (Greiner), covered by fine sieves
- slides
- small brush
- Petri-dishes
- microscopes
- shaker
- magnifying-glass

The fine sieves are for covering the vials in which the sections are freely floating, so that solutions can be poured out without losing the sections.

The brush is used for picking the floating sections up.

Reagents, solutions for step 3

- 1% H_2O_2-solution in 100% methanol
- PBS
- PBS with 2% goat serum and 0.5% Triton X 100
- PBS with Triton X 100 (0.5%)
- primary IgY-antibody in working dilution

- species-specific POD-labeled secondary antibody in working dilution
- 15 mg DAB (Sigma) in 50 ml PBS with 1500 ml ammonium nickel sulphate (3% w/v) and with 15 ml H_2O_2 (1 ml 30% H_2O_2 in 30 ml PBS)
- gelatine
- alcohol series
- entallan (Merck)

Procedure

Preparation of the fixative — Step 1

There are several possibilities:

- The use of tissue-samples already fixed (often by means of formalin), and the preparation of tissue-sections by a cryomicrotome or vibratome.
- The use of samples already fixed after transfer into paraffin, with the 'paraffin-dependence' of the primary antibody being a problem, and the preparation of tissue-sections by a microtome.
- Intracardial perfusion of an anaesthetized animal (rat) with a solution of fixative, followed by point 1 or 2.

The intracardial perfusion of an anaesthetized animal with Bouin's sublimate solution as a fixative (Sofroniev, 1987) is described below.

For further fixative solutions see Cuello 1983 and Ambrosius and Luppa 1987.

Bouin's sublimate solution is made as follows:

1. Add 1 l boiling distilled water to 200 g picrid acid, to form a saturated solution.
2. Concentrated solution of formaldehyde (37%).
3. Make a saturated aqueous sublimate solution (mercuric chloride, $HgCl_2$ [6% w/v in distilled water], dissolve the sublimate in a boiling water-bath).

4. Glacial acetic acid (to be added just before use).
5. Filter the single solutions.
6. Mix the solutions 1, 2, 3 and 4 just before use in the ratios 15:5:2:1 e.g. 315 ml, 105 ml, 42 ml, 21 ml.

Step 2 Cardial perfusion of a rat

1. Anaesthetise a rat with chloralhydrate (400 mg/kg body weight, i.p.).
2. Open the rat's rib-cage.
3. Cut off the tip of the heart and push a button cannula into the left ventricle as far as the aorta and fix the button cannula so that the heart-opening is shut.
4. Clamp off the body circulation with an appropriate clamp (Clamping the Vena cava and the Aorta descendens below the diaphragm cuts the greater circulation off, so the fixative is pumped solely through the pleural/brain circulation).
5. Open the right heart-vestibule, so as to ensure that the fixative can flow out.
6. Perfuse the anaesthetised rat with about 200 ml Haemaccel/PBS-solution (1:5 v/v), to rinse the blood away, using a pressure of 50-100 ml/min applied by an Ismatec MC 360 pump.
7. Perfuse with about 500 ml Bouin's solution, using the same pressure as before.
8. Prepare the brain.
9. Fix once more for 2 h in Bouin's sublimate solution.

Step 3 Visualisation of the antigen with IgY-primary antibody

See Figure 12 for an indirect immunohistochemical marking of antigen.

1. Cut the brain using a cryo-microtome or vibratome (we use a vibratome, the thickness of the sections is 50 μm).
2. Put the sections freely floating in PBS.

3. Wash the sections 3 x 15 min freely floating in PBS (in vials, Greiner), then put the vials onto a shaker, as also in the following steps.
4. Incubate the sections for 30 min freely floating in a 1% H_2O_2-solution in 100% methanol, repressing the non-specific peroxidase-reaction.
5. Repeat step 1.
6. Incubate for 30 min in PBS with 2% goat serum and 0.5% Triton X 100, blocking the non-specific binding sites.
7. Repeat step 1.
8. Incubate overnight for about 16 h at room temperature in primary IgY-antibody. (In this case we used the antibody Ch 22 diluted 1:500 with PBS-Triton X 100, see Figure 13).
9. Repeat step 1.
10. Incubate for 2 h in the species-specific POD-labeled secondary antibody (from donkey), diluted with PBS-Triton X 100).
11. Repeat step 1.
12. Stain the sections for about 10 min or less in a filtered DAB solution (15 mg DAB in 50 ml PBS), add 1500 µl ammonium nickel sulphate (3% w/v) and add 15 µl H_2O_2 (1 ml 30% H_2O_2 in 30 ml PBS).
13. Stop the reaction with distilled water.
14. Mount the sections on gelatine slides coated through the following procedure: Dissolve 1 g gelatine (type A or B, Sigma) in 50 ml distilled water boiling in a bath, add a pinch of chromalum, coat the slides with this solution, then dry the slides at 60°C for about 12 h.
15. Dry the sections at room temperature.
16. Transfer the sections into a rising alcohol series and leave them for 10 min in each of the following solutions respectively:
    - 30% alcohol
    - 50% alcohol
    - 70% alcohol

- 90% alcohol
- 96% alcohol, 1
- 96% alcohol, 2
- isopropanol
- xylol

17. Cover the sections with entellan (Merck).

Results

Fixing the tissues is a decisive step which may influence the results. In determining peptides, we have had good experiences with Bouin's solution, as have other authors (Ambrosius and Luppa 1987, Sofroniew 1983). As second Ab we use an anti-chicken IgY Ab from donkey, Dianova (703-035-155).

See Figure 16 a-f (see also Schade 1991).

Troubleshooting

Non-specific markings are very problematic. To avoid non-specific staining, it is advisable to dilute the primary or secondary antibodies with PBS, which contains normal serum (about 2%) from other species like goats, sheep and horses.

A precondition for a good result is a good fixation. A critical point is the formaldehyde solution, which may oxidize with increasing usage. In the case of frozen sections, the preservation of tissue-structure can be a problem.

The staining-solution should be prepared just before use and the reaction takes place in the dark. The sections are then blue-black. Take care that the DAB is not to old. Dissolved DAB should have a slight violet gleam.

We have achieved good results with this fixing and staining method. For further variations see Chapter 8, References.

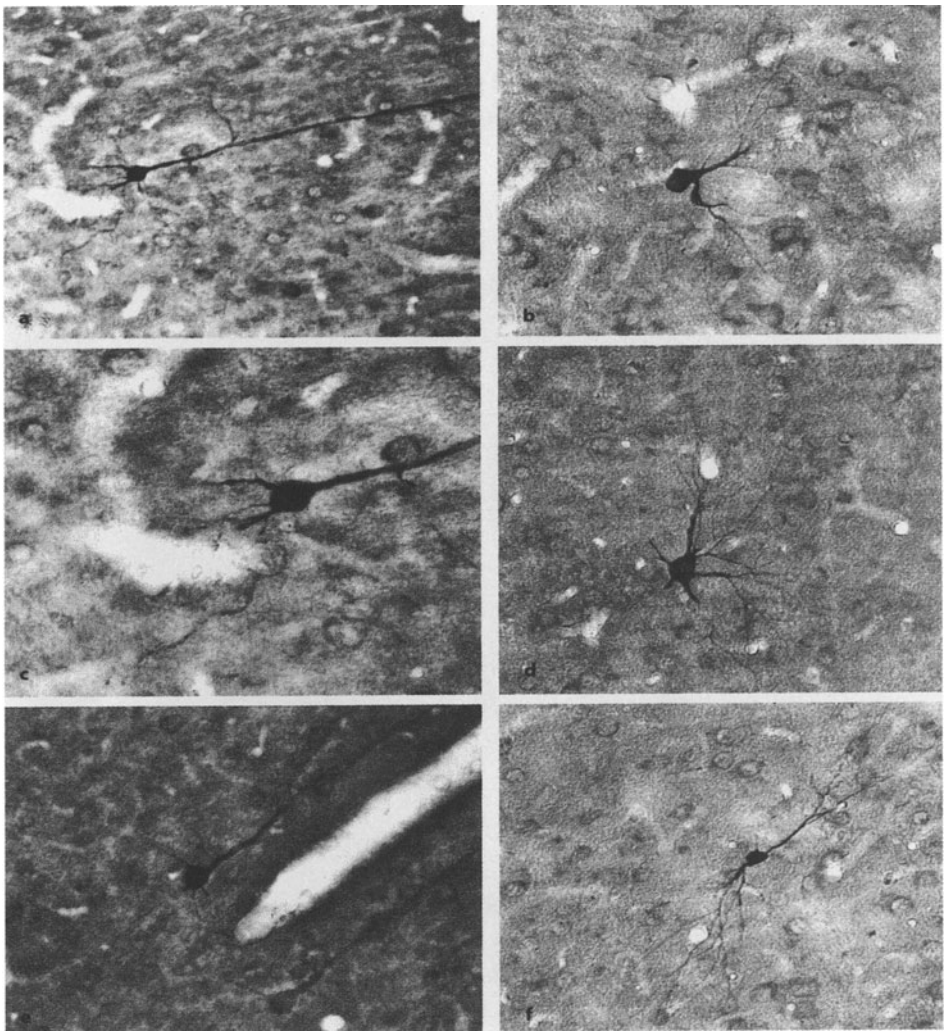


Fig. 16. Indirect visualisation of neurons with CCK-like immunoreactivity in the cortex and hypothalamus of the rat brain. As a primary antibody, an IgY anti-CCK antibody (Ch 22) was used. As an antigen, a MAP-CCK8 construct was used, which makes coupling to a protein carrier unnecessary (see Figure 13). As a secondary antibody, a commercial POD-marked antibody from donkey (Dianova) was used. In Figures 15a, c, the pyramidal neurons in the cortex are visualized. In Figure 15e, two neurons are marked with different degrees of intensity. This was not part of the method but is a result due perhaps to various degrees of activity of the neurons. Figures 15b, d, f show three different kinds of neurons from the thalamus of rats. Cortical pyramidal neurons have so far been successfully detected only twice (Sakamoto et al. 1984, Morino et al. 1994). The detection in the former case has been ignored in the relevant literature, and the detection in the latter case succeeded only after preliminary treatment with colchicin and an antibody developed against pro-CCK. We have two IgY antibodies (antigen: CCK8-derivatives) which reliable visualize pyramidal neurons.

Subprotocol 22
Immunohistochemistry Detection of MHC Class II-Antigens in Cryostate-Slices From the Lung Lymph-Nodes of Pigs Using Mouse Monoclonal Antibodies

Outline

See Figure 17.

Materials

See Subprotocol 21, equipment and materials for step 3, additionally:

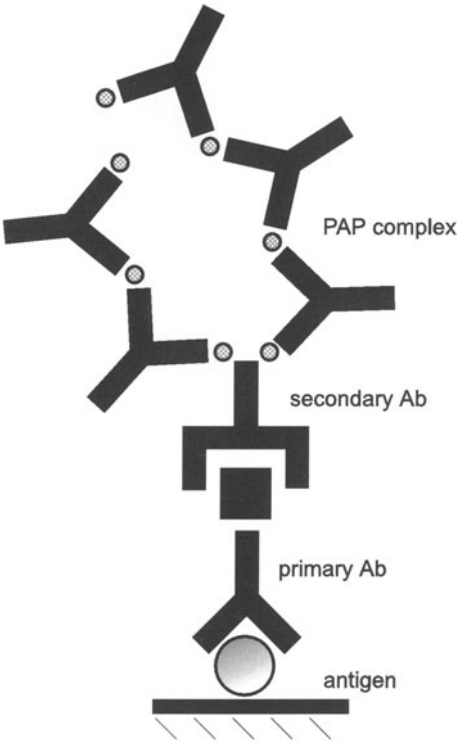


Fig. 17. Indirect marking through the PAP-technique (PAP = peroxidase-anti-peroxidase).

- frozen sections of lung lymph-nodes from pig (6-8 μm)
- PBS (pH 7.4)
- mouse mAb (MSA3, 2G6: isotype IgG)
- POD-labeled chicken anti-mouse IgG
- peroxidase-anti-peroxidase-complex (PAP-complex: SIGMA) diluted 1:800
- 3,3'-diaminobenzidine (DAB: Merck): 1 mg/ml PBS
- Canada balsam (Riedel de Häen)

Procedure

1. Prepare tissue sections as above using a cryostate.
2. Fix the air-dried sections in acetone for 10 min at room temperature.
3. Incubate with primary antibody for 30 min in a moist chamber at room temperature. To safe Ab-solution the sections may be encircled with a DAKO Pen (DAKO). In this way a barrier is formed which keeps the Ab-solution within the encircled area.
4. Wash thrice with PBS.
5. Incubate with POD-labeled secondary antibody (hen anti-mouse immunoglobulin) for 30 min in a moist chamber at room temperature.
6. Wash thrice with PBS.
7. Incubate with the PAP-complex.
8. Wash thrice with PBS.
9. Visualise the enzyme-linked antibody by having it react with DAB and hydrogen peroxide as described above and in Subprotocol 21 at room temperature for 10 min.
10. Counterstain the sections slightly with haematoxylin.
11. Mount them with Canada balsam.
12. Examine them by light microscopy.

Results

See Figure 18.

Troubleshooting

See Subprotocol 21.

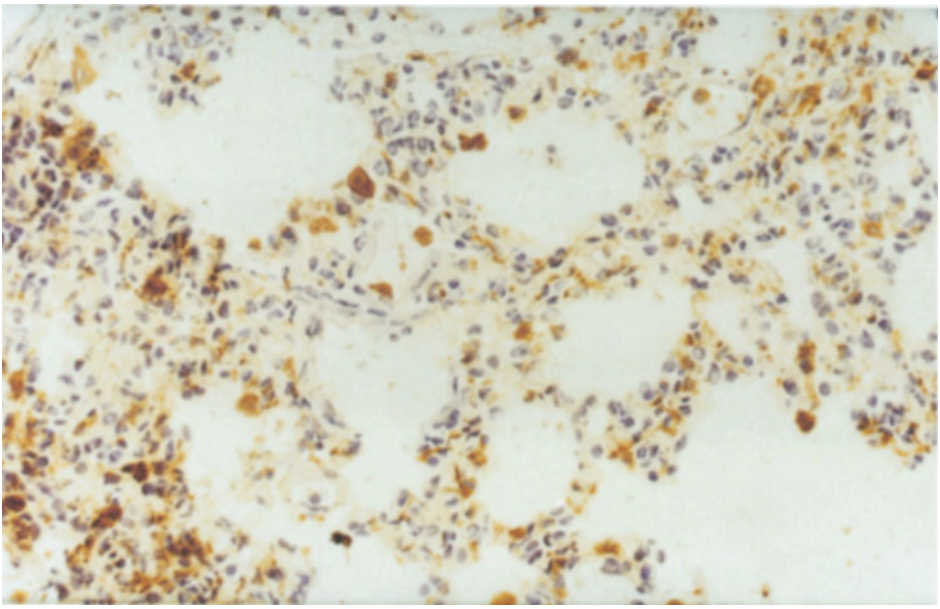


Fig. 18. Demonstration of MHC class II antigens on macrophages (lung lymph-nodes of a pig) according to Subprotocol 22. The brown colored cells are MHC class II positive.

Subprotocol 23
Detecting GABAergic (g-Amino-Butyric Acid) Cells in the Brain of Mongolian Desert Mice (*Meriones ungulatus*)

Outline

In this assay the chicken Ab is used as a non-labeled species-specific secondary Ab which is detected by a POD-labeled tertiary rabbit anti-chicken Ab.

Materials

See Subprotocol 21, additionally:

- brain sections (cryostate, 50 µm)
- methanol, PBS, goat normal serum, BSA, Triton X-100, sodium azide, Tris buffer
- mouse anti-glutamic acid decarboxylase (GAD, Boehringer Mannheim)
- unlabeled chicken anti-mouse Ig, diluted 1:15
- rabbit anti-hen IgY (POD-labeled, Chemicon, Temecula) diluted 1:500
- DAB solution (0,02%) in TBS with 0.05% H_2O_2

Procedure

1. Take the sections free floating in PBS in multi-well plates.
2. Incubate the sections for 60 min at 4°C in a 1% H_2O_2 solution in PBS/methanol (1:1), suppressing the non-specific peroxidase reaction.
3. Wash the sections 3 x 20 min in PBS.
4. Incubate the sections for 60 min at room temperature in PBS with 2% goat normal serum and 5% BSA, 0.1% Triton X 100, 0.01% NaN_3 blocking the nonspecific binding sites.

5. Incubate for 2 days at 4°C with anti-GAD (diluted 1:5.000 in PBS, 5% BSA, 0.1% Triton X 100, 0.01% NaN_3).
6. Wash the sections 3 x 20 min in PBS.
7. Incubate for 150 min with chicken anti-mouse IgG (diluted 1:15 in PBS, 1% BSA, 0.01% NaN_3).
8. Wash the sections 2 x 20 min in PBS and 1 x 20 min in TBS.
9. Incubate for 60 min at room temperature with POD-labeled rabbit anti-chicken IgY (diluted 1:500 in TBS, 1% BSA).
10. Wash the sections 3 x 20 min in TBS.
11. Stain the sections for 15 min in DAB solution.
12. Wash the sections 2 x 30 min in TBS and overnight in PBS at 4°C.
13. Mount the sections on slides.
14. Dry the sections at room temperature.
15. Transfer the sections into a rising alcohol series and leave them for 2 min in each of the following solutions respectively: optal, xylol (2 x) (see also Subprotocol 21).
16. Cover the sections with entellan (Merck).

Troubleshooting

See Subprotocol 21.

Subprotocol 24
Immunocytochemical Detection of CD44 in Human Keratocytes in vitro

Outline

For principle see Figure 12.

Materials

See Subprotocol 21, additionally:

- primary culture praeputium, about 7500 cells/well in 16-chamber slides (The culture is kept at 37°C till subconfluence)
- hen normal serum
- TBS, BSA
- anti-CD44 (mouse monoclonal antibody J173, Immuno-Tech)
- Cy3-labeled hen anti-mouse Ig
- entallan (Merck)
- fluorescence microscope

Procedure

1. Keep the cells at 37°C till confluence.
2. Fix the cells for 9 min with methanol, for 1 min with aceton (-20°C) on slides.
3. Dry the slides.
4. Wash the slides for 10 min in 0.05 M TBS pH 7.6.
5. Block with hen normal serum (0.1% in TBS with 5% BSA) for 2 h at room temperature.

6. Add the primary Ab, anti-CD 44, diluted 1:30 in TBS with 5% BSA.
7. Incubate the slides overnight in moist atmosphere at 4°C.
8. Wash the slides 3 x 10 min in 0.05 M TBS.
9. Add the secondary antibody Cy3-labeled hen anti-mouse immunoglobulin diluted 1:100 in TBS with 5% BSA.
10. Incubate the slides overnight in moist atmosphere at 4°C.
11. Wash the slides 3 x 10 min in 0.05 M TBS.
12. Transfer the slides in a rising alcohol series (see Subprotocol 21, step 3).
13. Cover the slides with entallan (Merck).
14. Analyse the slides by means of a fluorescence microscope.

Troubleshooting

See Subprotocol 21.

Subprotocol 25
Use of IgY-Ab as Secondary Antibodies in Flow Cytometry: Immunofluorescent Staining of a Single Cell Suspension from Human Peripheral Blood to Detect Surface Antigens Through One-Colour Labeling (Indirect Method)

The following reactions show the use of hen antibodies as secondary ones, as well as the efficacy of IgY-antibodies as double ones (labeled and unlabeled) and as double sandwich ones. Flow cytometry is widely used for detecting the expression of cell-surface and intracellular molecules and is used mainly to measure the intensity of fluorescence.

Antibodies fluorescently-labeled can bind specifically to soluble antigens or cell-associated molecules. The direct and indirect immunofluorescence of cells can yield information about the antigen and the binding of antibodies. The antigen-antibody

complexes can be detected by means of secondary antibodies coupled to fluorescent stains.

Flow cytometry data are generally analysed to type and define different cell populations and/or to ascertain the relative amounts of cell-surface antigen-molecules.

The frequency is determined by the analysis of single parameter fluorescence (histograms, see Fig. 20) and of dual-parameter fluorescence (dot points, see Fig. 22). The direct immunofluorescent method is faster but less sensitive than the indirect one. Many distinct antibodies (e.g. mouse ones) can be detected with the latter.

In indirect staining the primary reagent is not labeled but detected by a second fluorochrome-labeled reagent. This second reagent may be an antibody reacting specifically to the first one. Alternatively the streptavidin/avidin system can be used, where an antibody is conjugated to biotin and detected with phycoerythrin-labeled streptavidin/avidin.

Outline

See Figure 19.

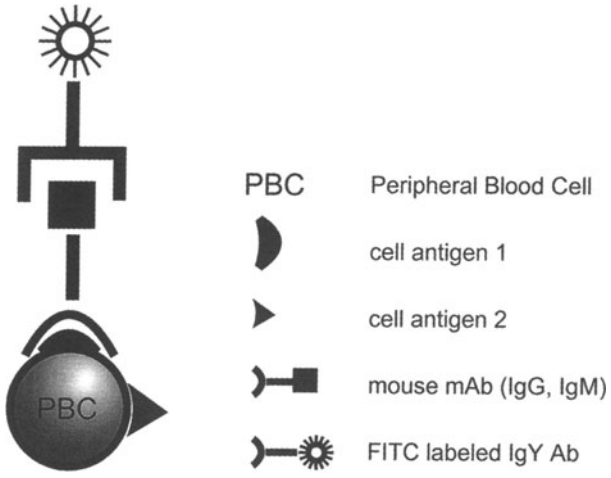


Fig. 19. Scheme of indirect marking of peripheral blood cells (PBC) for FACS analysis.

Materials

Equipment

- polystyrene microtitre plates (96, V)
- pipette and pipette tips
- centrifuge with rotor for microtitre plates
- vortexer
- FACscan (Becton Dickinson)
- refrigerator

Reagents, solutions

- living cells, like peripheral human lymphocytes
- PBS
- PBS/0.01 M sodium azide (washing solution)
- antibodies:
  - mouse monoclonal antibody in appropriate dilution
  - FITC-labeled hen anti-mouse IgY in appropriate dilution

Procedure

1. Add 50 µl unlabeled mouse antibody to the well on the plate.
2. Add 50 µl cell suspension (2-10 x 10^6/ml)
3. Mix gently.
4. Incubate for 45 min at 4°C in a moist atmosphere.
5. Wash the cells thrice with a wash buffer (50 µl, 100 µl, 100 µl) at 2 min intervals and each time centrifuge at 300 g for 3-5 min after washing.
6. Stain the cells with 50 µl FITC-labeled hen anti-mouse Ig.
7. Incubate for 30 min at 4°C in a moist atmosphere.
8. Wash the cells thrice as above.
9. Fix the cells, using 100 µl 1% formaldehyde/wash buffer.
10. Keep the cell suspension at 4°C until analysed by flow cytometry.

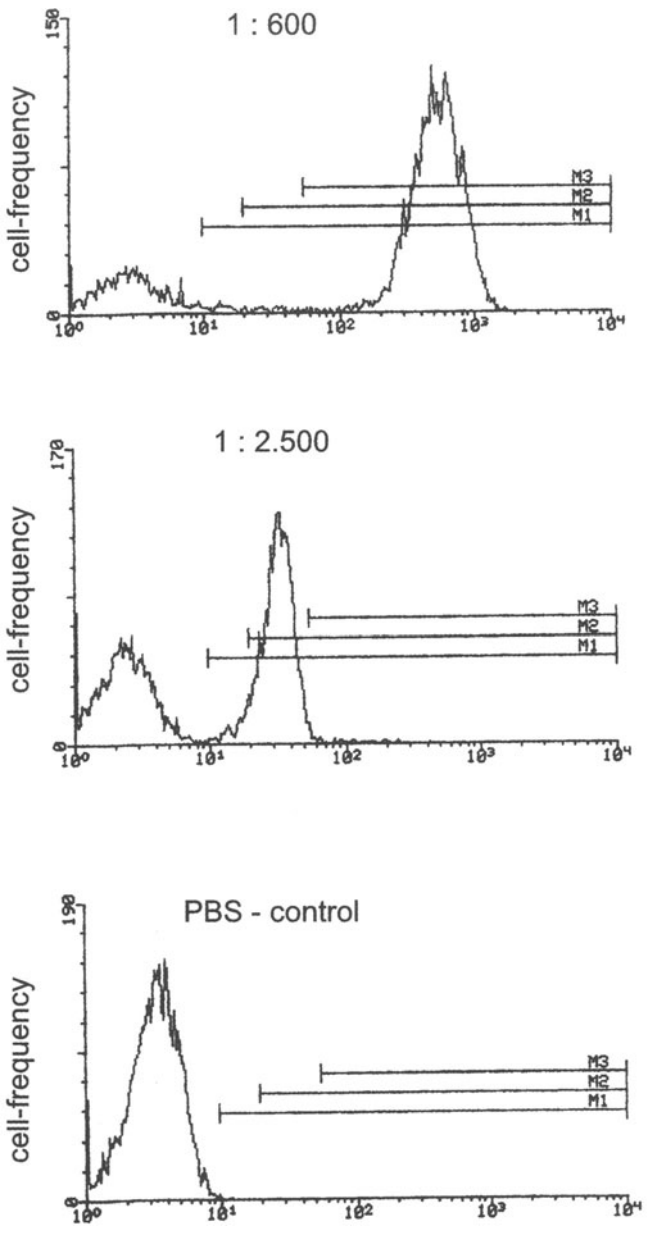


Fig. 20. Histogram of FACS-analysis to detect mouse mAB using biotin- or FITC-labeled chicken anti-mouse antibodies (see also Figure 21).

Results

See Figure 20.

Troubleshooting

Note that there is no loss of cells during the wash procedures. The resuspension after washing is performed merely by gentle lateral knocking on the microtiter plates. The analysis of the labeled and fixed cells can be performed in one week.

Subprotocol 26
Double-Immunofluorescent Staining of a Single Cell Suspension From Human Peripheral Blood to Detect Surface Antigens Through Two-Colour Labeling (Indirect/Direct Method)

Outline

See Figure 21.

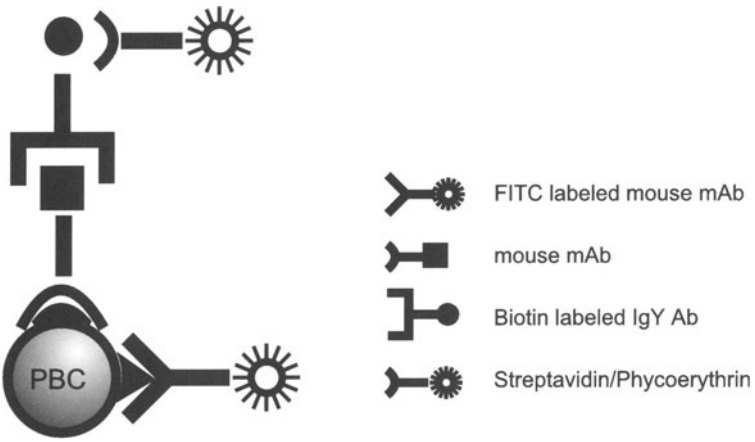


Fig. 21. Scheme of direct/indirect marking of PBC for FACS analysis.

Materials

See Subprotocol 25 (except antibodies).

Antibodies

- unlabeled mouse antibody (IgM, directed against CD4, B40, Ia/1) in appropriate dilution
- biotin-labeled hen anti-mouse IgM/streptavidin-phycoerythrin
- FITC-labeled mouse antibody (IgG, directed against CD3) in appropriate dilution

Procedure

1. Add 50 µl unlabeled mouse antibody (IgM) to the wells on the plate.
2. Add 50 µl cell suspension (2-10 x 10^6/ml).
3. Mix gently.
4. Incubate for 45 min at 4°C in a moist atmosphere.
5. Wash the cells thrice with a wash buffer (50 µl, 100 µl, 100 µl) at 2 min intervals and each time centrifuge at 300 g for 3-5 min after washing.
6. Suspend the cell-pellet again gently.
7. Incubate the cells with 50 µl hen-anti mouse IgM-biotin.
8. Incubate for 30 min at 4°C in a moist atmosphere.
9. Wash as above.
10. Add 50 µl labeled streptavidin-phycoerythrin.
11. Incubate for 30 min at 4°C in a moist atmosphere.
12. Wash as above.
13. Add 50 µl FITC-labeled monoclonal mouse antibody (isotype IgG).
14. Incubate for 30 min at 4°C in a moist atmosphere.
15. Wash as above.

16. Fix the cells, using 100 µl 1% formaldehyde/wash buffer.
17. Keep the cell-suspension at 4°C until analysed by flow cytometry.

Results

See Figure 22.

Troubleshooting

See Subprotocol 25.

Subprotocol 27
Double-Immunofluorescent Staining of a Single Cell Suspension from Human Peripheral Blood to Detect Surface Antigens Through Two-Colour Labeling (Indirect/Indirect Method)

Outline

See Figure 23.

Materials

Equipment See Subprotocol 25.

Reagents, solutions See Subprotocol 25 (except antibodies, specificity of the mouse mAb as in Subprotocols 25 and 26).

Antibodies
- unlabeled mouse monoclonal antibody (different isotypes, e.g. IgG1, IgG2b or IgM)
- FITC-labeled hen-anti mouse IgG1
- biotin-labeled hen-anti mouse IgG2b or IgM
- streptavidin-phycoerythrin in appropriate dilution

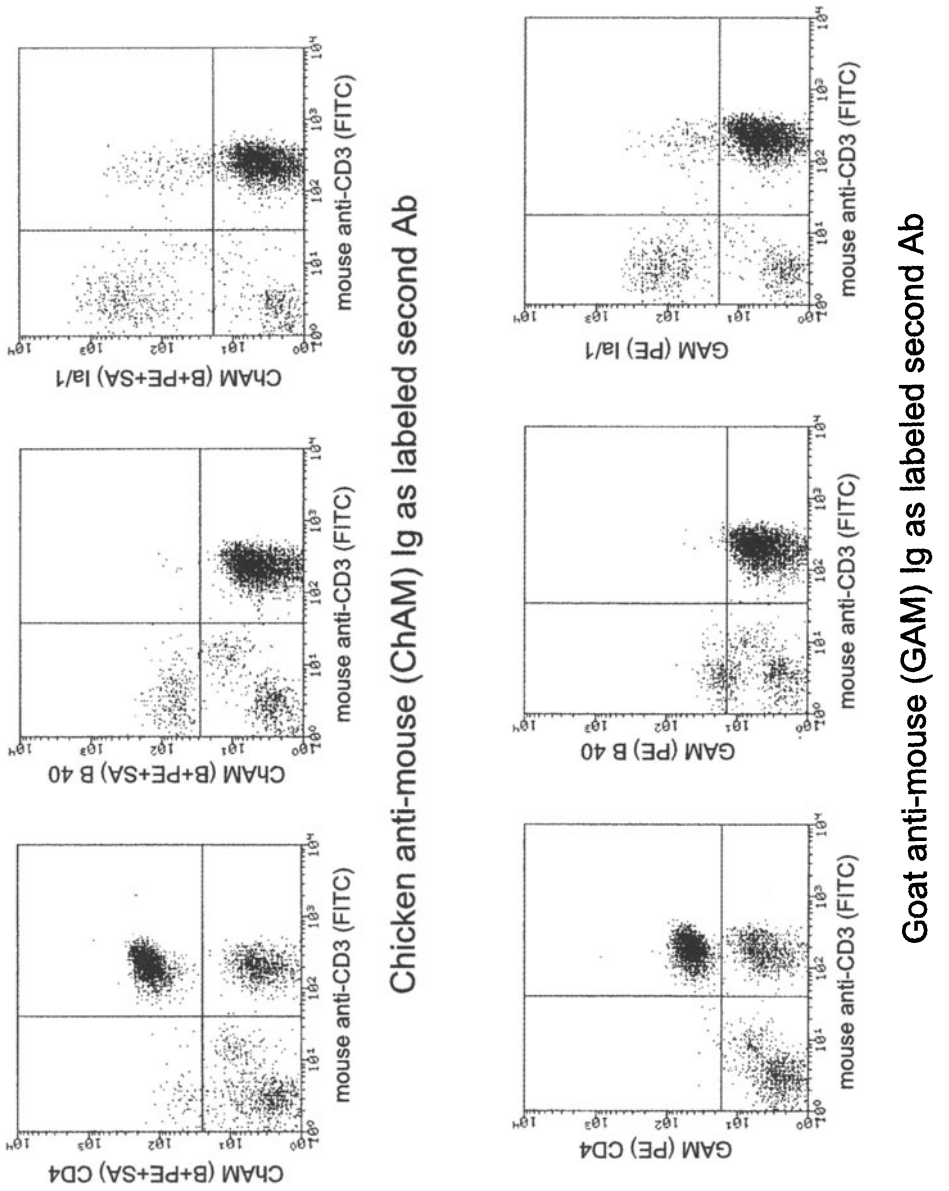


Fig. 22. Dot-plot of FACS-analysis to demonstrate several human cell differentiation antigens by means of the direct/indirect method. Comparison between the efficacy of an avian and a mammalian antibody.

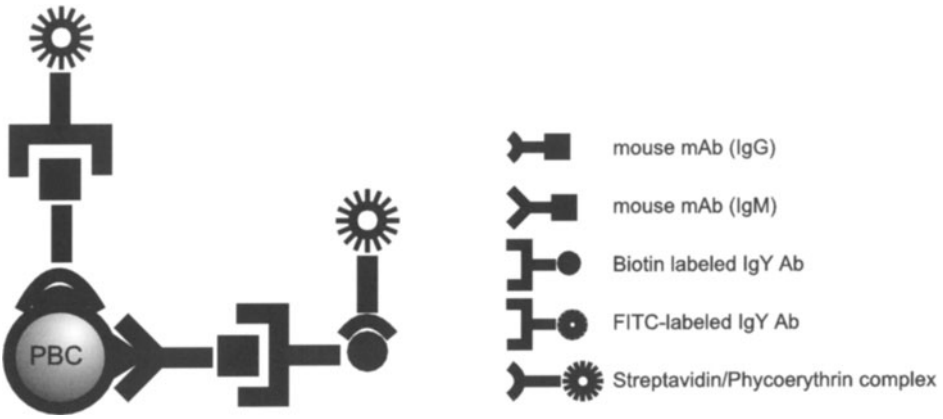


Fig. 23. Indirect/indirect marking of PBC for FACS-analysis.

Procedure

1. Add 50 µl unlabeled mouse mAb (IgG1) to the wells on the plate.
2. Add 50 µl cell suspension (2-10 x 10^6/ml).
3. Mix gently.
4. Incubate for 45 min at 4°C in a moist atmosphere.
5. Wash the cells thrice with a wash buffer (50 µl, 100 µl, 100 µl) at 2 min intervals and each time centrifuge at 300 g for 3 min after washing.
6. Suspend the cell pellet again gently.
7. Stain the cells with 50 µl FITC-labeled hen-anti mouse IgG1.
8. Incubate for 30 min at 4°C in a moist atmosphere.
9. Wash as above.
10. Add 50 µl unlabeled mouse mAb (IgG2b or IgM).
11. Incubate for 30 min at 4°C in a moist atmosphere.
12. Wash as above.
13. Add 50 µl biotin-labeled hen-anti mouse IgG2b or IgM.
14. Incubate for 30 min at 4°C in a moist atmosphere.

15. Wash as above.
16. Add 50 µl streptavidin-phycoerythrin.
17. Incubate for 30 min at 4°C in a moist atmosphere.
18. Wash as above.
19. Fix the cells, using 100 µl 1% formaldehyde/wash buffer.
20. Keep the cell-suspension at 4°C until analysed by flow cytometry.

Troubleshooting

See Subprotocol 25.

Subprotocol 28
Competitive Radioimmunassay Determination of Neuronal CCK-8 (Cholecystokinin Octapeptide)

In radioimmunoassays a reaction-partner is radioactively marked, and here the antigen (CCK) is marked with ^{125}I. By measuring the radioactivity of the reaction-product (Ag-Ab complex), conclusions can be drawn about the amount or concentration of the substance investigated. As with ELISA there are competitive and non-competitive assays. In the former, a marked and a non-marked substance compete for a limited number of binding sites, and in the latter a substance reacts with a marked binding-protein.

Outline

See Figure 24.

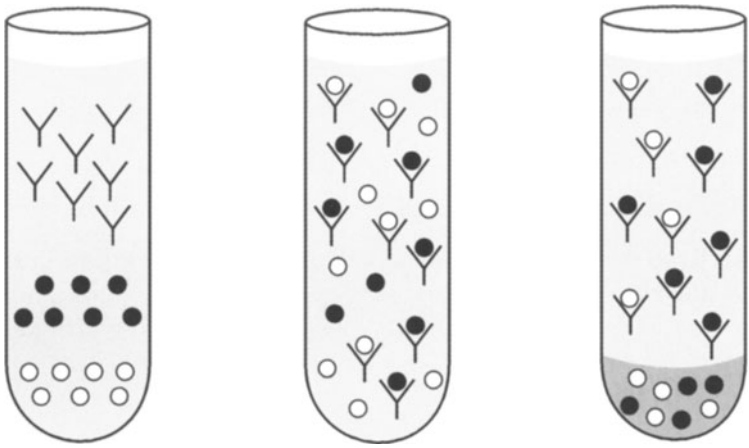


Fig. 24. A competitive radio immunoassay, taking as an example a peptide (CCK)-determination. The amount of the antibody (IgY anti-CCK antibody) and of the tracer are defined. The concentration of the samples varies. The more CCK contained by a sample, the less the marked tracer can be bound, and the lower is the sample's radioactivity.

Materials

Equipment

- vortex
- refrigerator
- cool-centrifuge (e.g. Hermle Z 360 K, rotor 220.27 V03)
- g-counter (e.g. 1470 Wizard, Pharmacia)
- pipettes
- pipette-tips
- safety-equipment for radioactive-work

Reagents, solutions

- RIA buffer: see Chapter 8
- plastic tubes (volume ca. 1 ml, compatible with the g-counter used)
- samples and standard (see Table 2)
- tracer (radio-labeled, adjusted to 1.000 cpm/100 ml)
- anti-CCK IgY-antibody diluted with RIA buffer (1:1.000)
- charcoal (Norit A, Serva) in RIA buffer

Procedure

1. Prepare the IgY-antibody dilution with RIA buffer.
2. Add the following solutions in a plastic tube (see Table 3):
   - Standard or sample
   - RIA buffer
   - Tracer (^{125}I-CCK)
   - Diluted IgY-antibody
3. Shake the mixture, using a vortex.
4. Incubate for 24 h or longer (see Chapter 8) in a refrigerator at 4°C.
5. Separate the bound from the unbound tracer by adding 500 µl of the charcoal mixture to the solutions in the tube.
6. Shake vigorously.
7. Incubate for 1 h in a refrigerator at 4°C.
8. Centrifuge the tubes for 20 min in a cooling- centrifuge at 4,000 cycles/min.
9. Decant the supernatant completely, pouring it out into another plastic tube.
10. Measure the supernatant with a γ-counter for 3-10 min.

Table 2. Example of a standard curve

Standard 6	0.2 fMol/100 µl	100 µl
Standard 5	1.0 fMol/100 µl	50 µl
Standard 5	2.0 fMol/100 µl	100 µl
Standard 5	4.0 fMol/100 µl	200 µl
Standard 4	10.0 fMol/100 µl	50 µl
Standard 4	20.0 fMol/100 µl	100 µl
Standard 3	80.0 fMol/100 µl	100 µl

The various volumes are equalised by means of the RIA buffer (Standard + RIA buffer = 200 µl). The standard curve is calculated with the RIA Calc Program (LKB, Wallac) as is the precision profile, which yields information about the most precise area of measurement (see Figure 25).

Table 3. Example of an RIA pipetting-scheme on the basis of an IgY-antibody

	Standard or sample	RIA-buffer	Tracer ^{125}I-CCK	Antibody	Charcoal
Non-specific binding	-	300 µl	100 µl	-	500 µl
Specific binding	-	200 µl	100 µl	100 µl	500 µl
Total	-	800 µl	100 µl	-	-
s6	100 µl	100 µl	100 µl	100 µl	500 µl
s5	50 µl	150 µl	100 µl	100 µl	500 µl
s5	100 µl	100 µl	100 µl	100 µl	500 µl
s5	200 µl		100 µl	100 µl	500 µl
s4	50 µl	150 µl	100 µl	100 µl	500 µl
s4	100 µl	100 µl	100 µl	100 µl	500 µl
s3	100 µl	100 µl	100 µl	100 µl	500 µl

Results

Through addition of active charcoal, the bound radioactivity is separated from the unbound (see Figure 24). Other means of separation are possible (e.g. separation by means of species-spe-

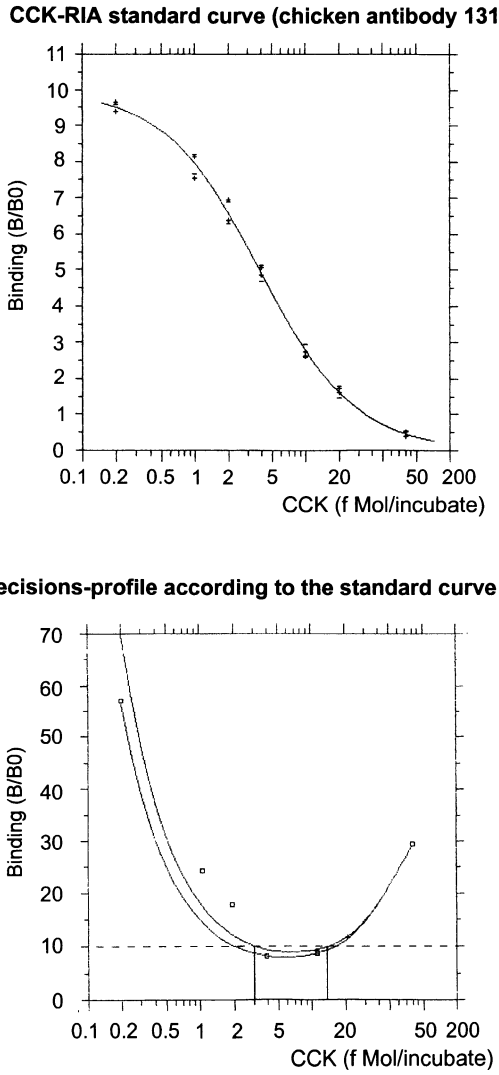


Fig. 25. An example of a standard curve (upper part). In the lower part is the corresponding precision-profile, which shows in which range measurements are most reliable. Here it is from about 3-15 fMol/incubate.

cific antibodies). For standard curve and precision profile see Figure 25 (Schade et al. 1988, Schade et al. 1996, Vick 1995).

Troubleshooting

There may be an increasing non-specific binding with increasing age of the tracer (decay period). Depending on the quality of the antibody used, an incubation-period of more than 24 h may be needed. A pre-incubation of antibody and sample may raise the sensitivity of the sample. A good separation of the bound from the unbound tracer may depend on the charcoal used.

Subprotocol 29
Complement Fixation Test (CFT)

In its latest edition of the O.I.E. "Manual of Standards for Diagnostic Tests and Vaccines" ("Office International des Epizooties", Paris 1996), CFT is second only to ELISA as a prescribed or recommended way of diagnosing infections. Its reliability, in diagnosing brucellosis for instance, has established it as a confirmatory test.

Complement (C) belongs to the non-specifically acting defense mechanisms which by itself doesn't act as an antibody but is needed for destruction of antigens by increasing phagocytosis or by extracellular lysis. In serum, the complement system (C1 - C 9 with additional subfactors) is present in an inactive form. Activation of C is caused by the formatiòn of immune complexes (IC) where the first C-fraction "C1q" joins the IC and thereby activates the cascade of subsequent C-fractions.

Outline

In vitro, the CFT provides an answer to the question whether C has been fixed by an antibody-antigen complex (IC) (positive case) or not (negative case). It is obvious that the quantity of C plays an important role in regard to the outcome of the

test. Therefore, C from the side of the test-serum has to be inactivated by heating to 56 - 60°C. For the test, heat inactivated C is replaced by a foreign properly diluted C collected normally from guinea-pig.

The test runs in two steps: During the first step, C is either fixed to the IC (positive), or not (negative), and is consequently either not available anymore or still available. During the second step, the resulting availability is tested by a second IC where sheep red blood cells (SRBC) together with anti-SRBC ("amboceptor" or "anti-sheep-hemolysin") form the hemolytic or indicator system. In a positive case, no C is available to cause lysis - here hemolysis- during the second step, and vice versa (Figure 26).

After binding to antigens, antibodies from hens are unable to activate the first factor (C 1) of guinea-pig complement and release the C-cascade (Benedict and Yamaga in Marchalonis J: Comparative Immunology, Blackwell Scient Publ 1976). During research work on CFT with avian sera, it was found that the simplest way to overcome this handicap was to add normal hen-serum to guinea-pig C which caused lysis of sensitized SRBC. Apparently, the corresponding C-components of hens and guinea-pigs are compatible (Brumfeld et al. 1961, Marquardt and Newman 1971).

Anti-complement reactions, in part very disturbing, led to further research. Stolfi et al. (1971) used an amboceptor from hens, guinea-pig C minus C 1, and C 1 from chicken to supplement the C1-devoid guinea-pig C. This modified CFT has been applied by Van Regenmortel and Burckhard (1985) for detection of IgY-antibodies directed against rotavirus and plant-virusses ("tomato bushy stunt virus" and "tobacco mosaic virus").The procedure was optimized by adjustment of the dilution-buffer during the procedure.

Materials

Equipment

- photometer (for photometric measurement of hemolysis or for subjective determination of the amount of the non-lysed erythrocytes)
- refrigerator

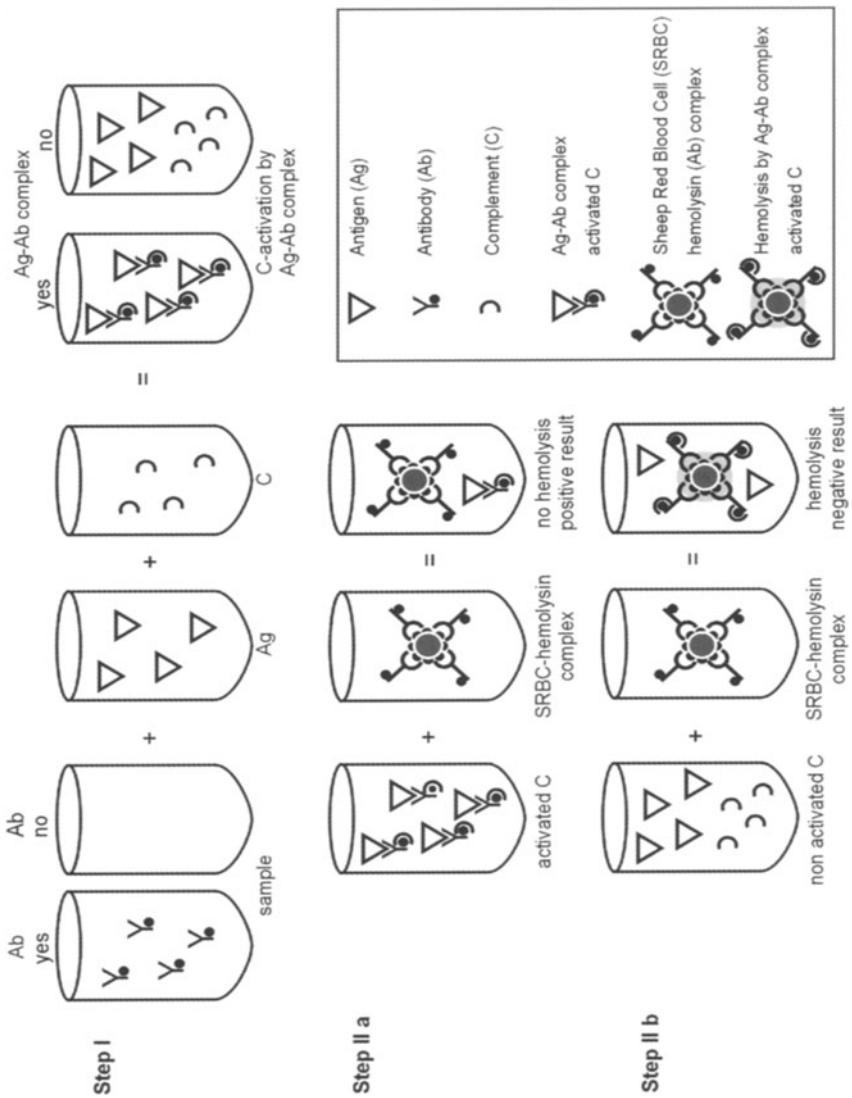


Fig. 26. The scheme of the complement fixation test. Stage I represents the stage of complement activation through the formation of the Ag-Ab complex (IC). If there are no antibodies in the sample to be determined, there is also no complement binding. Stage IIa shows full complement binding, so that no C can bind to the SRB hemolysin complex and consequently no hemolysis appears. This means that the sample contains the sought antibody. In stage IIb, there is unbound C, which binds to the SRBC-hemolysin complex and causes hemolysis. This means that the sample does not contain the sought antibody.

- micro-titre plates
- pipettes
- centrifuge
- waterbath 37°C
- waterbath 56 - 60°C
- CFT-buffer
- antigen
- antibody
- sheep red blood cells (SRBC)
- amboceptor (antibodies against SRBC)
- complement (from guinea-pigs and hens)

Reagents, solutions

Yolk-antibodies were extracted in the way described by Polsen et al. (1980).

- veronal-buffer (VB) pH 7.3 - 7.4

Buffers

85.000 g	NaCl
3.750 g	sodium 5.5-diethyl barbiturate
0.220 g	$CaCl_2 \cdot 2\ H_2O$
1.015 g	$MgCl_2 \cdot 6\ H_2O$
5.750 g	5.5-diethyl barbituric acid

Dissolve the acid hot and add the other components, then top the mixture up to 2000 ml (dist. water) to produce a fivefold concentrated buffer.

Preparation of the components

Dilute concentrated VB 1:5 and add 0.1% gelatin.

A1: GVB (gelatin veronal buffer)

Mix equal volumes of GVB and a 5% solution of dextrose in water, while doubling the concentrations of Ca^{++} and Mg^{++}.

A2: DGVB (dextrose GVB)

A3:DG (dextrose-gelatin) Mix 5% dextrose in dist. water with 0.1% gelatin, 0.00015 M Ca^{++} and 0.0005 M Mg^{++}.

B: Hemolytic system

B1: IgY anti-SRBC Ab (Amboceptor) Immunise a hen several times with washed SRBC (see Chapter 3), collect serum, mix it with an equal amount of glycerol and store it at -20°C.

B2: Sensitized SRBC (Eab^{ch}) Take washed sheep blood-cells (SRBC $1x10^8$) in 0.01 M isotonic EDTA in GVB without Ca^{++} and Mg^{++}, and sensitize with the same volume of non-activated IgY anti-SRBC diluted 1:100 in the same buffer and incubated for 30 min at room temperature. Wash the sensitized cells and adjust them to a concentration of 10^8/ml with dextrose GVB for immediate use.

C1: Chicken C 1 Precipitate the C 1-component of hen-C from the whole serum by addition of 1 volume of serum to 8 volumes 0.02 M acetate buffer, pH 5.0, then dissolve the precipitate in tenfold the original volume of GVB.

C2: Guinea-pig C without C 1 Resuspend lyophilized guinea-pig C in GVB pH 7.4 then dialyse it against GVB 7.4 (I=0.04). Remove the resulting precipitate by centrifuging and discard it. The supernatant fluid contains all the C-components of guinea-pig serum save C 1. Test for the optimal concentrations of guinea-pig C minus C 1, of hen C 1 and of hemolysing IgY anti-SRBC with a checkerboard system.

Procedure

The test is performed in tubes kept in an ice bath. The following reagents, all diluted with GVB were added:

1. 0.2 ml of antigen, 0.2 ml of chicken C1 and 0.2 ml of specific IgY (specific against the antigen) at different dilutions, incubation overnight, 4°C.
2. The ionic strength of the mixture was lowered to I=0.075 by addition of 0.6 ml of DG (to preserve maximal C 1 activity).

3. 0.2 ml of freshly prepared sensitized SRBC were added, incubation at 30°C for 30 min.
4. Addition of 0.2 ml guinea-pig C devoid of C1, incubation for 2 hours, centrifugation and measuring the supernatant by photometer at wavelength 413 nm.

Controls

The following controls may be used too, see subsequent diagram, Table 4:

- antiserums in suitable dilutions + C without antigen
- antigen + C without antiserum
- two tubes for single and double C-doses without antigen or antiserum, so as to reveal the C-dose for complete hemolysis
- two tubes with dilution buffers, to measure or exclude spontaneous hemolysis

Results

Centrifuge the tubes and determine the absorbance photometrically at 413 nm, then express the result as% fixed C through the following formula:

[(A_{413} total lysis - A_{423} background) - (A_{413} test - A_{413} background)] : [(A_{413} total lysis) - (A_{413} background) x 100]

The CFT is used only for research purposes but not for routine diagnostic work.

Troubleshooting

Using this test system it is very important to carry out careful controls according to Table 4 and to test out appropriate dilution ratios of the several components involved in reaction.

Table 4. Control schedule (Data in ml)

	IgY-Ab dilution (several)	Chicken C1	Antigen	Single dose C1	Double dose C1	Buffer
1.	0.2	0.2	-	-	-	0.2
2.	-	0.2	0.2	-	-	0.2
3.	-	-	-	0.2	-	0.4
4.	-	-	-	-	0.2	0.4

Subprotocol 30
Field Studies of the Prophylactic use of Specific Egg-Antibodies Against Diarrhoea due to Various Infections in New-Born Calves

Yolk antibodies in the prophylaxis and therapy of diarrhoea in domestic animals

Diarrhoea due to infection is one of the commonest dangers to animal-life world-wide. The standard therapy is with antibiotics, but the side-effects are notably negative, and the main ones are often negative too. Antibiotic therapy may harm the balance of bacterial species in the intestines, leaving the latter with a predisposition towards further infections, so it should be used to treat only feverish general ailments. In treating diarrhoea, priority should be given to rehydration and electrolytic supply.

Moreover, as a veterinary prophylaxis against diarrhoea in suckling calves and piglets, the dams are now given vaccinations, which via colostrum induce local passive immune protection in the sucklings' guts. Immune protection there is also significantly increased by means of specific antibodies administered orally. The sources of such antibodies may be serum, colostrum, milk and monoclonal antibodies.

An alternative source may be hens' eggs (Lösch et al. 1986), since serum IgY antibodies produced after immunization are transferred through egg-follicles to yolks (Rose et al. 1974, Kowalczyk et al. 1985). Hence IgY from yolk can be produced easily and abundantly, with a yield of up to 250 mg per hen's egg (Er-

hard et al. 1997). Akita and Nakai (1993) described a total IgY of 8 - 20 mg IgY/ml yolk with up to 10% of specific IgY. Specific IgY is also able to bind or to neutralize pathogens.

The efficacious use of colostrum- and milk-antibodies against diarrhoea had already been described, so it seemed advisable to investigate the efficacy of specific yolk-antibodies in prophylaxis and therapy. Due to the phylogenetic gap between birds and mammals the spectrum of antibodies may be quite different. Birds' may deal with epitopes which have no effects as antigens on mammals.

To check the practical efficacy of orally administered egg-antibodies, the investigations described under the following Subprotocols were carried out.

Outline

- Investigation of 2 experimental groups: **Experiment 1**
  - Group 1: E coli K99- and rotavirus-specific egg-powder (antibody-powder 22 g)
  - Group 2: control egg-powder from non-immunized hens (control-powder 22 g)
- Treatment with 22 g of egg-powder per day in the first 14 days post natum (p.n.) twice a day with the meal.
- Investigation of 3 experimental groups: **Experiment 2**
  - Group 1: E coli K99- and rotavirus-specific egg-powder (antibody-powder 16 g)
  - Group 2: E. coli K99- and rotavirus-specific egg-powder (antibody-powder 8 g)
  - Group 3: without feed additive (control)
- Treatment with altogether 16 or 8 g of egg-powder per day in the first 10 days p.n. twice a day with the meal.
- Investigation of 4 experimental groups: **Experiment 3**
  - Group 1: E coli K99- and rotavirus-specific egg-powder (antibody-powder 8 g)
  - Group 2: E coli K99- and rotavirus-specific egg-powder (antibody-powder 4 g)
  - Group 3: E coli K99- and rotavirus-specific egg-powder (antibody-powder 2 g)
  - Group 4: without feed additive (control)

- Treatment with altogether 8, 4 or 2 g of egg-powder per day in the first 14 days p.n. twice a day with the meal.

Procedure

In prophylaxis with specific egg-antibodies, attention has to be paid to not only the range but also the times of possible infection. Calves most often have diarrhoea in the first two weeks of life. The main pathogens are rotaviruses, coronaviruses, K99-positive enterotoxic E. coli and cryptosporides. The pathogen diagnosis was normally carried out on day 7, or in the case of diarrhoea, with the help of Lactovac^{8}-ELISA (Hoechst Unterschleißheim, Germany). The parameters considered are the rate, intensity and duration of diarrhoea, as also the gain in bodyweight. Altogether, three investigations were carried out independently of one another. In the egg-powder, titres of antibodies were measured against E. coli K99 of at least 330 ELISA-units and against rotaviruses of at least 800 units by the neutralization test. The egg powder was fed with the colostrum or milk.

1. Immunization of laying hens (see Chapter 3).
2. Obtaining egg-powder by spray-drying.
3. Testing the powder for pathogen-specific yolk-antibodies for use in calves and piglets.
4. Pathogen diagnosis of the infected animals.
5. Prophylactic treatment of the infected animals by applying the egg-powder (together with colostrum or milk).
6. Monitoring the development of the infection by means of various parameters (rate, intensity and duration of diarrhoea, body weight gain).

Results

The field trials of the prophylactic use of hen-antibodies on calves were carried out in three different places: south-west Germany, south-east Germany and Turkey.

Table 5. Prophylactic use of specific egg-antibodies against diarrhoea due to various infections in new-born calves

Parameter	22g Ab (trial 1)	22g Co (trial1)	16g Ab (trial 2)	8g Ab (trial 2)	Contr. (trial 2)	8g Ab (trial 3)	4g Ab (trial 3)	2g Ab (trial 3)	Contr. (trial 3)
All calves	n=51	n=54	n=58	n=60	n=55	n=55	n=53	n=56	n=80
Rate of diarrhoea (%)	53	69	69	68	75	15*	15*	14*	39
Duration of diarhoea (days)	42*	60	48*	53	64	55*	67*	48*	84
High intensity of diarrhoea (%)	31	49	5	5*	23	nd	nd	nd	nd
Daily gain in body weight (g/d)	431*	269	378*	367*	276	292*	300*	269*	185
Rotavirus positive									
Rate of diarrhea (%)	80	76	76	78	94	19*	44	28	60
Duration of diarrhoea (days)	4458	44*51	6858	7248*	88				
High intensity of diarrhoea (%)	19	50	5*	7*	50	nd	nd	nd	nd
Daily gain in body weight (g/d)	462	254	311*	300*	122	277	238*	246*	154
E. coli K99 positive									
Rate of diarrhea (%)	31*	92	25	33*	100	nd	nd	nd	nd
Duration of diarrhoea (days)	60	68	24	54	55	nd	nd	nd	nd
High intensity of diarrhoea (%)	25	50	0	0	20	nd	nd	nd	nd
Daily gain in body weight (g/d)	462*	262	556*	333	189	nd	nd	nd	nd

Calves received egg-powder in amounts ranging from 2 to 22 g with (Ab) or without (Co) pathogen-specific egg-antibodies. No egg powder was applied in trial 2 and 3 (Co). In trial 3 the intensity of diarrhoea was not determined, and the calves were too few for results related to Escheria coli K99 to be significant (*$p<0.05$ vs. control groups of the corresponding trial.).
nd = not determined

In the case of the parameters rate, intensity and duration of the diarrhoea, the results of using antibody-powder were often significantly different to the results of using a control-powder or no feed additive. Large differences were recorded in the gain in body-weight. The most important results are summarised in Table 5 and described in more detail by Erhard et al. (1993) and Özpinar et al. (1996).

Subprotocol 31
Field Studies of the Prophylactic Use of Specific Egg-Antibodies Against Diarrhoea Due to Infection in Weaned Piglets

Outline

3 experimental groups:

- Group 1: egg-powder with specific antibodies against E. coli K88, K99, 987P and rotaviruses (antibody-powder)
- Group 2: egg-powder of not immunized hens (control-powder)
- Group 3: without any feed additive (control)

Procedure

The study on weaned piglets was carried out in a piglet-producing farm in the south of Germany with 70 sows and their 179 weaned piglets.

To limit possible financial harm to the owner, animals whose health was strongly affected were treated additionally with antibiotics.

In the egg-powder the following antibodies were measured:

E. coli K88	(ELISA: at least 470 units)
E. coli K99	(ELISA: at least 100 units)
E. coli 987P	(ELISA: at least 860 units)
rotaviruses	(neutralization test: at least 512 units)

1. Suckling of the piglets by the sows in the 7th week of life.
2. Use of the respective powder from the 28th to the 56th day of life. The powder was offered in a feed ration made up of 5% of the mixture and was mixed freely.
3. Diagnosis of pathogens by investigating samples of feces.
4. Evaluation of the parameters: rate and duration of the diarrhoea and gain in body weight additional treatment with antibiotics in the event of severity of symptoms.

Results

Diarrhoea in suckling piglets was positively affected by the administration of specific egg-powder containing pathogen specific Abs. Whereas diarrhoea occurred in 60% of the piglets in the control group with egg-powder and in 57% in the control group without egg-powder, it occurred in only 17% of the group with antibody-powder.

The efficacy of the antibody-powder is likewise shown by the incidence, duration and intensity of disturbances to the general well-being of piglets in the respective groups. For instance, more than half the piglets in the control groups had to be treated with antibiotics. There were no significant differences in the intensity and duration of diarrhoea in the various groups nor in the gain in weight. The results are shown in Table 6 and described in more detail by Erhard et al. (1996).

Subprotocol 32
Field Study of the Therapeutic Use of Specific Egg-Antibodies Against Diarrhoea Due to Infection in Weaned Piglets

Outline

The therapeutic use of specific egg-antibodies was investigated on newborn piglets in two farms of south Germany which had problems with enterotoxic E. coli (ETEC) infections and diar-

Table 6. The prophylactic use of specific egg-antibodies against diarrhoea due to infection in weaned piglets

Parameters	Control-group (n=58)	Control egg-group (n=61)	Antibody-group (n=60)
Rate of diarrhea (%)	56.7	60.7	17.2*
Duration of diarrhea (days)	1.4 (SD 0.7)	1.5 (SD 0.8)	1.3 (SD 0.5)
High intensity of diarrhea (%)	62	68	60
Severity of symptoms (%)	26.7	39.3	5.2*
Daily gain in body weight (g)	310 (SD 50)	280 (SD 30)	290 (SD 50)
Additional treatment (%)	51.7	55.7	8.6*
Mortality rate (%)	1.7	3.3	0.0

The animals in the antibody-group or control egg-group were given egg-powder (5% of the ration), respectively with or without pathogen-specific egg-antibodies, whereas the piglets in another control group were given no egg-powder at all (*$p<0.05$ to the control groups, SD = standard deviation).

rhoea. The antibody-titres of egg-powder are according to the trial described under Subprotocol 31 (procedure).

Investigation of 3 groups:

- Group 1: given egg-powder with specific antibodies against E. coli K88, K99, 987P and rotaviruses (antibody-powder)
- Group 2: given egg-powder of not immunised hens (control-powder)
- Group 3: given no feed additive (control)

Procedure

1. Oral administration of the corresponding egg-powder with a dosage help (3 g per animal per day for 3 days after the start of diarrhoea).
2. Diagnosis of the pathogens.
3. Measurement of the parameters intensity and duration of the diarrhoea.

Table 7. Therapeutic use of specific egg-antibodies against diarrhoea due to infection in weaned piglets

	Control without powder	Control with powder	Control with antibody-powder
Farm 1	1.9	1.8	1.3
Farm 2	3.5	2.6	1.0

The numbers indicate the mean duration of diarrhea in days.

Results

Several samples of feces from piglets suffering from diarrhoea were examined, and hemolytic E. coli with the antigen K88 was found.

On the two farms, altogether 105 new-born piglets got diarrhoea.

On average, the diarrhoea lasted for the number of days as indicated in Table 7. Regarding the intensity of diarrhoea, there were clear differences between the results for piglets treated with antibody-powder and those for the controls. On the whole, diarrhoea occurred more often in the control groups and was more watery and continuous. The results are described in more detail by Kellner et al. (1994).

Comments

Schmidt et al. (1989) and Wiedemann et al. (1990) were able to show that yolk antibodies, especially in the form of the full egg, are well protected from the processes of digestion. Shimizu et al. (1988) found an acid-stability of purified yolk-antibodies up to pH 4.0. In young animals, whose digestive system is not yet fully functional, orally administered antibodies pass fairly freely through the intestine, so the precondition for the local effect of specific antibodies against diarrhoea due to infections is fulfilled, though little is still known about the concentrations of antibodies needed. A tip about use is given by Yokoyama et al. (1993) in reporting on an infection trial. The specific antibodies proved to be efficacious only if their period of passage through the intestines was long enough.

The advantage of this kind of immune prophylaxis or therapy is that effective action can be taken at any time. It is possible to react immediately and specifically to pathogens, as with a farm-specific vaccine. Care must only be taken to react adequately to a given spectrum of pathogens with a corresponding spectrum of egg-powder antibodies.

This method of local immune-protection helps limit the use of antibiotics to fewer cases and thus limit the development of resistance and imbalance among flora in the intestines. Moreover with the combination of colostral and egg antibodies, the range of antibodies is extended and the efficacy of treatment improved.

In all investigations, irrespective of the kind of animal, of the form in which the antibodies were administered as prophylaxis or therapy, and of the animal's age, the administration of pathogen-specific yolk-antibodies was notably effective (see also Peralta et al. 1994, Ikemori et al. 1996, 1997). An amount of antibodies low in comparison to the concentration of antibodies in the colostrum was enough to thwart pathogens.

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