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A Modification of the Indirect Immunofluorescence Test for Detection of *Ehrlichia phagocytophila* Antibodies

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Hardeng, F.: A modification of the indirect immunofluorescence test for detection of *Ehrlichia phagocytophila* antibodies. Acta vet. scand. 1991, 32, 499–502. – A modified technique for production of antigen and performance of the test is described. A suspension of infected neutrophils was directly applied to multiwell slides. Multichannel pipettes may be used for dilution and application of sera. The modification inreases the capacity both by production of the antigen and by performance of the test. This paper also gives a quantitative determination of the antibodies.

tick-borne fever.

Introduction

Tick-borne fever (TBF) is a rickettsiosis of sheep, goats, cattle, and deer caused by *Ehrlichia phagocytophila* (*Scott* 1984). The vector is the sheep tick, *Ixodes ricinus*. The disease is characterized by high fever and typical haematological changes. The diagnosis is confirmed by demonstration of the agent in Giemsa-stained phagocytosing white blood cells.

Four techniques have so far been described for detecting antibodies: direct fluorescence (*Tuomi* 1967), complement fixation (*Snodgrass & Ramachandran* 1971), counter immunoelectrophoresis (*Webster & Mitchell* 1988), and indirect fluorescence (*Paxton & Scott* 1989). The present method is a modification of the indirect immunofluorescent antibody (IFA) technique described by *Paxton & Scott* (1989). The method is less time consuming and can handle larger series of samples than the original method.

Materials and methods

Production of antigen

Whole blood was taken from 10 elderly ewes in a flock in south-eastern Norway, an endemic tick-borne fever area. One ml of blood from each ewe was pooled and injected iv into a sheep from a tick-free area. The inoculated sheep developed TBF. A stabilate was prepared by drawing heparinized blood on the 2nd day of parasitaemia, adding 10 % dimethyl sulphoxide, and immediately freezing at –76°C (*Foggie et al.* 1966).

For production of antigen 1 ml of the stabilate was thawed and inoculated iv into a male goat. The animal was selected because it exhibited an exeptional neutrophilia, and this was probably a response to pneumonia and lymphadenitis caseosa, these diseases being common in the herd from which the animal originated. The neutrophilia was considered as an advantage for the antigen formation. On the 2nd day of parasitaemia a

granulocyte enriched concentrate was prepared as described by *Carlson & Kaneko* (1972) with slight modifications. Fourty ml of blood was added 4 ml phosphate buffered saline (PBS pH = 6.8) with 1.5 % EDTA. The sample was divided into four parts containing 10 ml each which were centrifuged for 20 min at 1000 g. Plasma, buffy coat and the uppermost layer of packed erythrocytes were discarded. The packed erythrocytes were resuspended in 4 ml PBS, lysed by addition of 2 parts distilled water, and isotonicity was restored after 35–40 s with hypertone PBS. The suspension was washed twice. The final white cell pellets, which consisted mainly of granulocytes, were pooled in 1 ml PBS. The cell suspension was counted using a Coulter Counter, and PBS added to give a final concentration of 30×10^9 cells/l.

The granulocyte enriched suspension containing the antigen was spread onto multiwell teflonized slides by means of an applicator (*Christensson* 1986). The rods of the applicator were placed 0.5 cm apart, thereby making it possible to apply serum dilutions to the slides with a 5 channel pipette formerly used to dilute serum samples. The cell suspension was thoroughly mixed every 10 min during application. The antigen was applied to the slides within 3 h after the blood was withdrawn. The antigen slides were left to air dry, and then wrapped in lens cleansing tissues and aluminium foil and stored at –76°C in plastic bags with dry silicagel until use. Fourty ml of blood yielded 800 slides with 10 antigen wells each. The male goat was bled twice 4 h apart on the 2nd day of parasitaemia.

Blood was drawn from a male goat which had not been exposed to *E. phagocytophila* and control antigen spots were prepared.

Test sera

Negative control sera were obtained from 15 sheep originating from tick-free areas. These sera were diluted 1:64, 1:128 and 1:256 with PBS pH 8.0.

Positive sera were obtained from 10 lambs. They were inoculated iv with a stabilate of TBF at an age of 6 to 8 weeks. Rectal temperatures and blood smears were used for confirmation of diagnosis. The blood smears were also examined for the presence of *Eperytrozoon ovis*. Sera were collected before inoculation, and at 2, 6 and 15 weeks post inoculation (pi).

Serial 2-fold serum dilutions were prepared when the endpoint titre was to be established. The endpoint titre was defined as the highest dilution of serum giving fluorescent staining of TBF-bodies.

Test procedure

Antigen slides were removed from the freezer, left in room temperature for 5 min, and then fixed in cold acetone (–20°C) for 5 min. The antigen spots were flooded with serum dilutions, placed in a humidified chamber and incubated at room temperature for 30 min. Positive and negative control sera diluted 1:128 were included each time the test was done. The slides were rinsed 2×5 min in PBS pH 8.0. Fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-sheep immunoglobulin was obtained from Dakopatts A/S, Denmark, and diluted 1:100 in PBS. The FITC-conjugate was flooded onto each well and incubated as above. The slides were rinsed 2×5 min in PBS, finally flushed in distilled water and allowed to dry. Cover slips were mounted with glycerol:PBS (pH 8.0) 9:1. The wells were examined in a UV-light-equipped microscope at a magnification of 400 ×. At least 30 cells in each well were examined.

Test for reproducibility

Three sera (low, intermediate and high antibody titre) were tested to end titre on 7 different days. The mean logarithmic titre was calculated.

Results

The control antigen prepared from the *E. phagocytophila*-negative animal did not show any fluorescing bodies, only a faint fluorescence being observed. The autofluorescence of granules in eosinophils was strong and easily differentiated from TBF-fluorescing bodies.

The negative control sera were negative in dilutions 1:128 an 1:256. However, serum from one of the sheep gave a faint fluorescent staining of TBF-resembling bodies in the cytoplasm at a dilution of 1:64, and because of the false positive reaction at this dilution, the dilution 1:128 was employed as the lowest positive titre.

The titre of sera from experimentally infected sheep were positive up to the dilution 1:16384. The results are expressed as reciprocal of serum dilution in Table 1.

Table 1. Reciprocal indirect immunofluorescent antibody titre in sera from experimentally infected lambs.

	Weeks post inoculation			
Lamb no.	0	2	6	15
1	Neg	8 192	16 384	4 096
2	128	16 384	16 384	16 384
3	256	2 048	4 096	2 048
4	Neg	8 192	16 384	4 096
5	Neg	8 192	16 384	4 096
6	Neg	8 192	16 384	4 096
7	Neg	8 192	16 384	16 384
8	Neg	4 096	4 096	4 096
9	128	16 384	16 384	2 048
10	Neg	8 192	8.192	4 096
Mean (logarithmic)		7 580	11 480	4 680
Median		8 192	16 384	4 096

The mean titre was calculated by 10-logarithm. Negative samples were given the value 1:64.

Superinfection with *Eperytrozoon ovis* was observed in lambs no. 3, 8 & 10. These 3 lambs showed the lowest titre by six weeks pi (Table 1).

The results from reproducibility testing for the low titre sample ranged from 1:128 to 1:256, mean 1:182; intermediate titre sample ranged from 1:512 to 1:2048, mean 1:1072 and the high titre sample ranged from 1:4096 to 1:16384, mean 1:9772.

Discussion

This indirect immunofluorescence antibody test detects a specific rise of the antibody titre within 2 weeks pi in experimentally infected animals. All 10 lambs still had antibodies 15 weeks pi. From the reproducibility test of the 3 positive sera it is shown that all results were within 2 doubling dilution steps. The advantage of this method, compared to the method described by *Paxton & Scott* (1989), is that the capacity is considerably increased, due to the marked reduction in the time required to prepare the antigen and to perform the test. The use of a multichannel pipette is possible.

Three lambs (no. 2, 3 & 9) had a low antibody titres before infection. The lambs were kept tick free before inoculation, but they originated from a flock affected by TBF, and these positive reactors were most likely due to the presence of maternal antibodies.

Some sera also gave a non-specific staining of cytoplasm in the cells. This phenomenon complicated the reading of the test, as such non-specific staining may conceal a positive staining of TBF-bodies if they are less intensely stained than cytoplasm. Non-specific staining became less of a problem when the

sera were diluted. Only a few samples in this material were troublesome when sera were diluted 1:128 or more. In field material this problem seems to be associated with certain flocks. It is possible that the non-specific staining is linked to certain strains of *E. phagocytophila*. This hypothesis has not, however, been investigated because of the lack of tools for differentiation of strains. The reduced antibody development seen in the Eperytrozoon ovis infected lambs has also been found for antibody responses to other antigens (*Larsen & Waldeland* pers. comm.).

The tested sera show an increase of titre to *E. phagocytophila* of 3 dilution steps or more within 2 weeks pi. The test may be an alternative way to diagnose tick-borne fever.

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Sammendrag

En modifikasjon av indirekte immunofluorescens test for påvisning av antistoffer mot Ehrlichia phagocytophila.

En modifisert teknikk for produksjon av antigen og utførelse av testen er beskrevet. En suspensjon med infiserte nøytrofile granulocytter avsettes direkte på objektglass med 10 teflonbrønner. Multikanal pipetter kan nyttes til fortynning og applikasjon av sera. Modifikasjonen øker kapasiteten både ved produksjon av antigen og ved utførelsen av testen. Artikkelen inneholder også en kvantitativ bestemmelse av antistoffer.

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