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HOST RESPONSE TO INFECTION

Sensitivity and specificity of an indirect enzyme-linked immunoassay for the diagnosis of *Brucella canis* infection in dogs

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The diagnosis of *B. canis* infection in dogs is based on bacteriological examination and serological methods including agglutination and gel diffusion tests. Bacteriological studies are the only methods that have been considered specific but, as intermittent periods of abacteraemia may occur, a negative blood culture cannot be used as a criterion for excluding canine brucellosis. Close contact between people and infected dogs increases the risk of transmission; however, its impact on public health is probably underestimated due to lack of reporting and inadequate diagnostic services. This paper describes an indirect enzyme-linked immunoassay (IELISA) procedure for the diagnosis of brucellosis caused by *B. canis* in a population of normal and infected dogs previously screened by the buffered plate antigen test (BPAT) and rapid slide agglutination test (RSAT). The serological survey was performed with 446 field sera. The 270 sera from the asymptomatic group found negative by BPA, RSAT and blood culture showed IELISA specificities of 96.7% and 100%, respectively, when cut-off values of OD 0.237 and 0.281 were selected. For 52 sera from culture-positive dogs, IELISA sensitivity was 100% with cut-off values of OD_{414} 0.237 and 0.281. OD_{414} 0.281 was selected because this value provided the highest accuracy with minimal false-negative and false-positive results. This cut-off value was used to study 124 blood culture-negative but RSAT positive sera. IELISA produced 107 positive results; the 17 sera that were negative by IELISA presented a wide range of reactivities by RSAT (2 were RSAT positive at 1 in 2 dilution and 15 were weakly positive with pure serum). These samples were probably from animals at an early stage of the infection or were false-positive results. The IELISA described here detects IgG and IgA antibodies that are useful for evaluating the clinical status of dogs. Although RSAT is a practical screening test, a supplementary technique such as IELISA should be used on all positive RSAT samples to ensure diagnostic specificity. Furthermore, people in contact with infected dogs could be investigated for possible transmission. The procedure described in this study was relatively simple and could have widespread applications.

Introduction

Transmission of *Brucella canis* commonly occurs by contact with products of abortion or subsequent vaginal discharges. Infected males harbour organisms in the prostate gland and epididymides for many months after the bacteraemia has ceased and may disseminate the disease in semen when they breed [1]. Clinical signs are not adequate to diagnose canine brucellosis, as many infected males are clinically normal. However, the infection may be suspected when there is a history of abortions, diskospondylitis or poor reproductive performance in either sex.

Serological tests are the methods most commonly used to evaluate the status of dogs before breeding or whenever brucellosis is suspected. Blood cultures are essential for diagnosis, especially if serological results are ambiguous. The serological tests usually used are the rapid slide agglutination test (RSAT) and the tube agglutination test (TAT) (both with 2-mercaptoethanol). These tests are sensitive but many false-positive results

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have been found. Agar gel immunodiffusion (AGID) has been used but sometimes the precipitin lines are difficult to interpret. More recently, indirect enzyme-linked immunoassay (IELISA) with cytoplasmic protein antigen, hot saline extracts or cell-wall antigens have been proposed [2–5].

Because of the threat of transmitting the disease to man, dogs suspected of having brucellosis should be investigated promptly. This paper describes an IELISA procedure for the diagnosis of brucellosis caused by *B. canis* in a population of normal and infected dogs previously screened by buffered plate antigen test (BPAT) and RSAT. The IELISA cut-off value was determined and the performance of the tests in terms of diagnostic specificity and sensitivity was analysed.

Materials and methods

Serological tests

The RSAT screening tests were performed as described previously [3], but with serial serum dilutions to find the final titre. Briefly, 10 μl of serum dilution were mixed with 10 μl of antigen on a 25 × 75-mm glass slide for 1–2 min and results were read with a 10× microscope objective, including a control standard serum whose titre was known. The antigen was prepared at ANLIS Dr C. G. Malbrán from the strain (M−) variant of *B. canis* kindly provided by Professor L. Carmichael who also supplied the antigen used as reference (L. Carmichael, personal communication). The buffered plate agglutination test (BPAT), Rose Bengal test (RB) and plate agglutination test (PAT) were done as described previously [6] with an antigen prepared at ANLIS Dr C. G. Malbrán from *B. abortus* strain 1119-3.

IELISA

Antigen. The antigen was obtained from the (M−) variant of *B. canis* by the procedure described previously for *B. ovis* [7, 8]. Briefly, *B. canis* saline extract was prepared as described by Myers *et al*. [9], then centrifuged at 254 000 *g* in a Kontron Instrument Ultra Centrifuge in a TFT 45.94 rotor for 4 h at 4°C. The pellet was dissolved in PBS, pH 7.2, frozen at −20°C and used at 1 in 2000 dilution after OD_{414} readings of various antigen dilutions with strongly positive, weakly positive and negative sera as controls. The control sera were from dogs with positive isolation of *B. canis* and positive RSAT with a titre of 1 in 128 and 1 in 8, respectively. The negative serum was from a healthy dog negative in RSAT and BPAT.

Conjugate. A lyophilised horseradish peroxidase-conjugated protein A/G was obtained from Immuno-Pure (Pierce Lb) and used at 1 in 20 000 after titration with strongly positive, weakly positive and negative dog sera.

Procedure. The antigen diluted in 0.06 M sodium carbonate buffer (pH 9.6) was passively coated on to polystyrene plates (Nunc 2-69620, Denmark) at 50 μl/well and incubated for 18 h at room temperature and then washed five times in 0.01 M phosphate-buffered saline (PBS) containing Tween 20 0.05%, pH 7.2 (PBS/T). Control and test sera were added at 1 in 100 in PBS/T, 50 μl/well, for 1 h at room temperature. After five washes in PBS/T, appropriately diluted horseradish peroxidase-conjugated protein A/G was added, 50 μl/well, and incubated for 1 h at room temperature. After five washes in PBS/T, the final step was the addition of 100 μl of chromogenic substrate (4.0 mM H_2O_2 and 1.0 mM 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid) diammonium salt in 0.05 M citrate buffer, pH 4.5) per well. The plate was shaken continuously on an orbital shaker and, after 10 min, the OD was measured at 414 nm in a photometer (Labsystems Multiskan EX microplate reader) with 100 μl of chromogenic substrate in a plate as a control for the microplate reader. When the test is positive, colour develops.

Bacteriological studies

Brucella organisms were isolated as described previously [1] from a blood culture, semen or vaginal discharge. The isolates were typed as recommended by the International Committee on Systematic Bacteriology (ICBN) Subcomittee on Taxonomy of the Genus *Brucella* [10] at ANLIS Dr C. G. Malbrán.

Canine sera

The 446 sera included in the study were divided into the following groups. (i) *Brucella*-infected dogs: 52 sera were from dogs with confirmed brucellosis by clinical examination, serology and positive cultures. (ii) Asymptomatic dogs: 270 sera were from healthy dogs with no clinical or epidemiological evidence of brucellosis plus negative blood culture and negative serological tests such as RSAT and BPAT. (iii) Dogs with suspected brucellosis: 124 sera from dogs with clinical symptoms compatible with brucellosis, showing positive results to RSAT at any titre but negative blood culture.

Data analysis

Diagnostic specificity and sensitivity were determined initially with 95% confidence limits by plotting the data for negative and positive samples on a frequency histogram. The data were subsequently analysed by receiver-operator characteristics (ROC) analysis [11].

Results

The 270 sera negative in IELISA had a mean OD_{414} value of 0.148 (SD 0.043). Fig. 1 shows the frequency

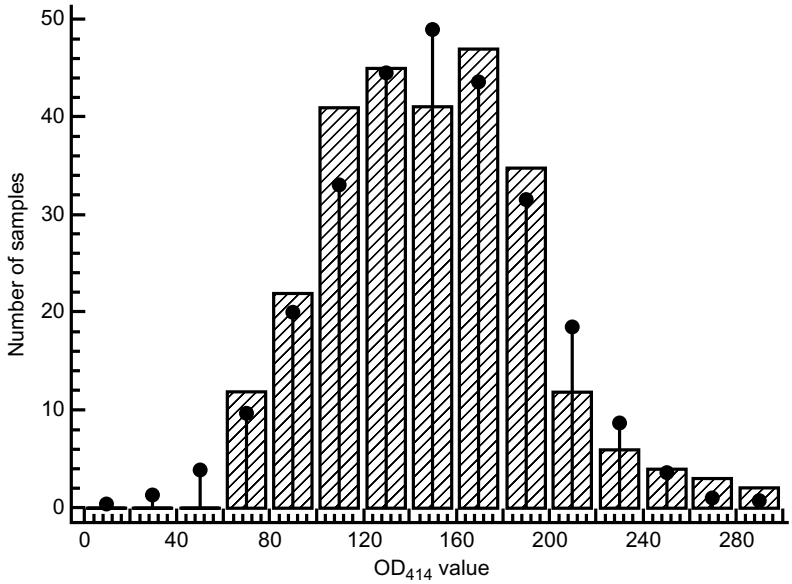

Fig. 1. Frequency distribution of IELISA results with 270 serum samples negative for antibody to *B. canis*.

distribution of these sera. Therefore, a cut-off value of OD_{414} 0.236 (2 SD) or OD 0.279 (3 SD) was established and then confirmed with 52 positive sera from *Brucella*-infected dogs and 270 negative sera from asymptomatic dogs by ROC curve (Fig. 2). The OD_{414} 0.281 cut-off value resulted in IELISA sensitivity and specificity of 100%.

Table 1 shows the serological test results for the sera of 52 dogs with positive isolation of *B. canis*; most of the isolates were from blood culture except dog 46, which was positive by swab of vaginal discharges. Of the 124 sera from dogs suspected of having brucellosis, six with weak or positive BPA titres were studied by PAT and RBT. Only one was weakly positive in RB and PAT at a dilution of 1 in 50. All six sera were positive in both IELISA and RSAT. Of 118 sera negative by BPA, 101 were positive by IELISA, showing low, moderate or high titres by RSAT. Of the 17 sera negative in IELISA, 2 were positive by RSAT at a dilution of 1 in 2 and 15 were weakly positive as pure serum.

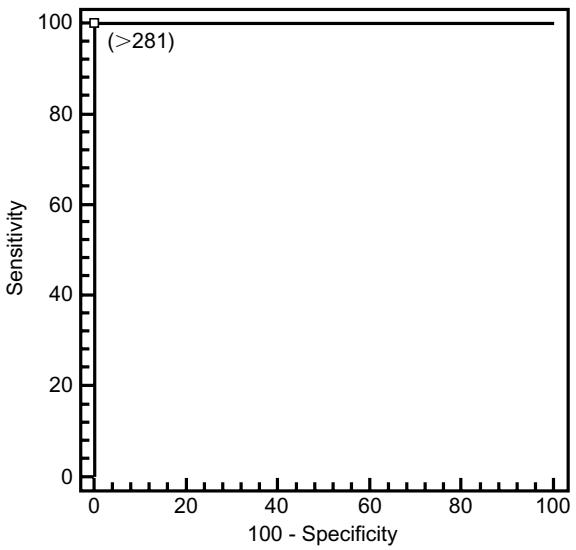

Fig. 2. ROC analysis plotting percent sensitivity (y axis) against 100-specificity (x axis) for various cut-off values. From the data a cut-off value of OD_{414} 0.281 provides specificity and sensitivity values of 100%.

Discussion

Canine brucellosis is an insidious disease that may be suspected when abortions occur in the last trimester or when epididymitis and testicular atrophy are observed in male dogs. These may be infertile and may show orchidism with varying degrees of prostatitis. However, many infected dogs are clinically normal. Close contact between man and infected dogs increases the risk of transmission; however, the impact on public health is probably underestimated because of lack of reporting and inadequate diagnostic services. The diagnosis of *B. canis* infection in dogs is based on bacteriological examination and serological methods, usually agglutination and gel diffusion tests. Bacteriological studies are the only method that has been considered specific but, as intermittent periods of abacteraemia may occur, a negative blood culture cannot be used as a criterion for excluding canine brucellosis. Agglutination tests have good sensitivity but their lack of specificity and the occurrence of false positive serological results make a specific test necessary.

The objectives of this study were to ascertain the usefulness of IELISA for the diagnosis of brucellosis caused by *B. canis* and to determine the cut-off value. The serological survey was performed with 446 field

Table 1. Serological response of sera from *B. canis* culture-positive dogs* to IELISA and RSAT

Serum no.	RSAT†	IELISA (OD_{414})	Isolated from
1	128	0.930	Blood
2	8	0.749	Blood
3	8	0.762	Blood
4	16	0.687	Semen and blood
5	4	0.531	Blood
6	64	0.977	Blood
7	32	0.691	Blood
8	8	0.938	Blood
9	32	0.673	Blood
10	64	0.751	Blood
11	32	0.902	Blood
12	8	0.961	Blood
13	8	0.982	Semen and blood
14	4	0.898	Blood
15	2	0.503	Blood
16	4	0.870	Blood
17	8	0.548	Blood
18	2	0.567	Blood
19	2	0.562	Blood
20	8	0.582	Blood
21	32	1.070	Blood
22	4	0.494	Blood
23	2	0.405	Blood
24	32	0.838	Blood
25	16	0.653	Blood
26	4	0.706	Blood
27	4	0.729	Blood
28	8	0.746	Blood
29	2	0.502	Blood
30	4	0.615	Blood
31	64	0.793	Blood
32	4	0.356	Blood
33	32	1.180	Blood
34	4	0.950	Blood
35	2	0.668	Blood
36	32	0.744	Blood
37	32	0.654	Blood
38	16	0.456	Blood
39	8	0.582	Blood
40	32	0.430	Blood
41	256	0.579	Blood
42	32	0.506	Blood
43	64	0.491	Blood
44	32	0.412	Blood
45	256	0.498	Blood
46	128	0.626	Vaginal swab
47	8	0.432	Vaginal swab and blood
48	32	0.574	Vaginal swab and blood
49	16	0.590	Blood
50	64	0.640	Blood
51	32	0.946	Blood
52	16	0.489	Blood

*All sera were negative for BPAT.
†Serum dilutions.

sera. The 270 sera from the asymptomatic group found negative by BPA, RSAT and blood culture showed IELISA specifications of 96.7% and 100%, respectively, when cut-off values of OD_{414} 0.237 and 0.281 were selected. For 52 sera from culture-positive dogs, the IELISA sensitivity was 100% with cut-off values of OD_{414} 0.237 and 0.281.

The cut-off value of OD_{414} 0.281 was selected because this value has the highest accuracy plus minimal false-negative and false-positive results. With this cut-off value, 124 blood-culture negative but RSAT-positive sera were studied and IELISA produced 107 positive results. The 17 sera that were negative by IELISA presented a wide range of reactivities by RSAT (2 were RSAT positive at a dilution of 1 in 2 and 15 were weakly positive with pure serum); these samples were probably from animals at an early stage of the infection or were false-positive results. Of six sera that were positive by both BPA and RSAT, only one was weakly positive in RB, but all six sera were strongly positive in RSAT and IELISA. Some overlapping in the detection of antibodies against rough and smooth *Brucella* strains has been reported [4], but in the present study this phenomenon was observed in only 6 (1.34%) of 446 cases.

The IELISA described here detects IgG and IgA antibodies that are useful for evaluating the clinical status of dogs. Although the RSAT is a practical screening test, a supplementary technique such as IELISA should be performed on all positive RSAT samples to ensure diagnostic specificity. Furthermore, it is suggested that studies of people in contact with infected dogs should be performed to investigate possible transmission.

The data presented clearly indicate that IELISA was more sensitive and specific than RSAT. The procedure described in this study was relatively simple to perform and may have widespread applications.

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