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# Diagnosis of human brucellosis caused by *Brucella canis*

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The transmission of *Brucella canis* to man commonly occurs through contact with infected dogs or their secretions, or through direct laboratory exposure. The disease is underdiagnosed due to a general lack of serological testing facilities and misconceptions concerning its prevalence. This report shows the potential use of an indirect ELISA (IELISA) for the diagnosis of human brucellosis caused by *B. canis* in a population of patients negative by smooth-*Brucella* antigen tests but positive by rapid slide agglutination test (RSAT). One hundred and ten sera from asymptomatic people found negative by tests using smooth *Brucella abortus* antigen and by RSAT showed an IELISA specificity of 100 % when a cut-off value of 27 % positivity (%P) was selected. For 17 sera from patients with positive *B. canis* culture or in close contact with culture-positive dogs, the IELISA sensitivity was 100 % with the same cut-off value. The positive patients presented clinical symptoms similar to brucellosis caused by other species of *Brucella* and some of them received antibiotic treatment and made good progress. Using this cut-off value, we studied 35 patients with negative blood cultures but positive RSATs, and IELISA detected 18 as positive; of the 17 IELISA-negative, two were RSAT-positive at dilution 1 : 2 and 15 were weakly positive with pure serum. These samples were probably from patients at an early stage of infection or indicate false-positive results. No cross-reaction was observed among the sera from nine cases with a diagnosis other than brucellosis, but cross-reactivity was evident in sera from patients infected with smooth-*Brucella* species. Since routine brucellosis diagnosis does not include *B. canis* investigation, infection with this species may be more widespread than is currently suspected. The RSAT could be a suitable screening test for the diagnosis of *B. canis* human brucellosis, and a supplementary technique, such as IELISA, performed on all positive RSAT samples that were negative by *B. abortus* antigen could ensure diagnostic specificity and confirm the diagnosis.

## INTRODUCTION

The most common modes of transmission of *Brucella canis* to man are through contact with infected dogs, which may disseminate the disease for many months after bacteraemia has ceased, through contact with their secretions and through direct laboratory exposure (Carmichael & Shin, 1996).

The disease is underdiagnosed due to general lack of serological testing facilities and misconceptions concerning its prevalence. Culture-positive cases have been reported in laboratory personnel, animal technicians and persons known to have close and frequent contact with infected dogs (Carmichael *et al.*, 1980).

Abbreviations: BPAT, buffered plate agglutination test; CELISA, competitive ELISA; CF, complement fixation; IELISA, indirect ELISA; RSAT, rapid slide agglutination test; TAT, tube agglutination test.

Human infections are probably more common than indicated in published reports, though serological methods and criteria for evaluating results vary greatly. The serological techniques most often used to detect *B. canis* antibodies in humans are the agglutination tests (Lewis & Anderson, 1973; Hoff & Schneider, 1975; Hoff & Nichols, 1974; Monroe *et al.*, 1975; Flores-Castro & Segura, 1976; Ying *et al.*, 1999; Polt & Schaefer, 1982). The infection was diagnosed by serological methods in a 17-month-old child, a woman with fever of unknown origin and a man with granulomatous hepatitis and splenomegaly (Tosi & Nelson, 1982; Rousseau, 1985; Schoenemann *et al.*, 1986). Complications such as mycotic aneurysms of the tibioperoneal arteries, aortic valve vegetations, calvarial osteomyelitis and more recently a presumptive case of *B. canis* endocarditis, diagnosed by serology at the Centers for Disease Control and Prevention (Atlanta), have been described (McKee & Ballard, 1999; Ying *et al.*, 1999; Piampiano *et al.*, 2000).

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As a national centre for human brucellosis our laboratory is engaged in the serological and bacteriological diagnosis of patients with symptoms and/or epidemiology compatible with this disease. On the basis that people could potentially be infected by *B. canis* we used serological and bacteriological methods to study patients with negative serological tests to smooth-*Brucella abortus* antigen.

Recently we reported an indirect ELISA (IELISA) test for the detection of antibodies to *B. canis* in dogs that has been demonstrated to be highly specific and sensitive (Lucero *et al.*, 2002). We now report the potential use of this IELISA for the diagnosis of human brucellosis caused by *B. canis* in a population of patients who tested positive by rapid slide agglutination test (RSAT) but negative by smooth *B. abortus* antigen.

## METHODS

**Human sera.** Sera from the 179 people included in the study were classified into five groups. The first group consisted of 17 sera from patients with positive *B. canis* culture or in close contact with culture-positive dogs. The second group of 110 sera was obtained from asymptomatic people with no clinical or epidemiological evidence of brucellosis, with negative blood culture and negative RSAT, buffered plate agglutination test (BPAT), tube agglutination test (TAT), complement fixation (CF) and competitive ELISA (CELISA) results. A third group, suspected of having brucellosis caused by *B. canis*, included 35 patients with clinical symptoms compatible with brucellosis and negative BPAT, TAT, CF, CELISA tests and blood culture but positive or weakly positive RSAT results. The fourth group included nine sera from patients with infectious diseases other than brucellosis supplied by the Bacteriology Department, INEI-ANLIS 'Dr C. G. Malbrán'. Another eight sera were from patients with brucellosis caused by smooth-*Brucella* species isolated and typed at our laboratory.

**Serological tests.** BPAT, TAT and CF were run as described previously (Lucero & Bolpe, 1998) with antigens prepared at ANLIS 'Dr C. G. Malbrán' using the *B. abortus* 1119-3 strain. CELISAs were run as previously reported (Lucero *et al.*, 1999); the antigen (S-LPS from *B. abortus* 1119-3) and the MAb were standardized and supplied by the Brucellosis Centre of Expertise and OIE Reference Laboratory, Animal Diseases Research Institute (ADRI), Canada. The conjugate pre-adsorbed with bovine, equine and human serum protein was from Jackson Lab.

**RSAT.** The RSAT was used as a screening test, run as described previously (Lucero *et al.*, 2002; Carmichael & Joubert, 1987) with serial sera dilutions in order to find the final titre. Briefly, 10 μl of serum dilution was mixed with 10 μl of antigen on a 25×75 mm glass slide for 1–2 min and the results were read under a ×10 microscope objective. A strong control serum with a known titre was also included. The 2-mercaptoethanol (2ME)-RSAT was performed by mixing 25 μl of serum dilution with 25 μl of 0·2 M 2-ME solution; after 1 min 50 μl of antigen was added and read in the same way. The antigen was prepared at ANLIS 'Dr C. G. Malbrán' with the strain (M−) variant of *B. canis*.

**IELISA.** The antigen was obtained from the (M−) variant of *B. canis* as described previously (Lucero *et al.*, 2002). Briefly, *B. canis* hot saline extract was prepared, then centrifuged at 254 000 ***g*** in a Kontron Instrument UltraCentrifuge 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 a 1 : 2000 dilution after $OD_{414}$ readings of various antigen dilutions using strongly positive, weakly positive and negative sera as controls. The strong control sera were from a patient who had positive haemoculture and a positive RSAT with a titre of 1 : 16, and the weakly positive control sera were from a patient who had a positive RSAT with a titre of 1 : 2 and negative haemoculture. The negative serum was from a healthy person with negative haemoculture and serological tests for both smooth and rough antigens.

A lyophilized horseradish peroxidase-conjugated protein A/G was from ImmunoPure (Pierce Lb.) and was used at 1 : 20 000 after testing various working dilution ranges with strongly positive, weakly positive and negative human sera.

The antigen diluted in 0·06 M sodium carbonate buffer (pH 9·6) was passively coated onto polystyrene plates (Nunc 2-69620, Denmark) at 50 μl per well, incubated for 18 h at room temperature and then washed five times in 0·01 M PBS containing 0·05 % Tween 20, pH 7·2 (PBS/T). Control and test sera were added at 1 : 50 in PBS/T, 50 μl per 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 per well, and incubated for 1 h at room temperature. After five washes in PBS/T, the final step was the addition of 100 μl per well of chromogenic substrate (4·0 mM hydrogen peroxide and 1·0 mM 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt in 0·05 M citrate buffer, pH 4·5). The plate was shaken continuously on an orbital shaker and after 10 min the $OD_{414}$ was measured in a photometer (Labsystems Multiskan EX microplate reader) with 100 μl of chromogenic substrate in a plate as a control for the microplate reader. The test is positive when colour develops. The standard control serum used on each plate makes it possible to convert the optical density reading to percent positivity (%P).

**Bacteriological studies.** *Brucella* organisms were isolated from three human blood cultures by inoculating 5 ml of blood into 25 ml of liquid medium. Only one blood culture was done on serum from each dog using paediatric bottle holding. The strains isolated were typed as recommended by the former ICBN Subcommittee on Taxonomy of the Genus *Brucella* (Corbel & Brinley-Morgan, 1984) at ANLIS 'Dr C. G. Malbrán'.

**Data analysis.** The strong control sera were from a patient who had positive haemoculture and positive RSAT with a titre of 1 : 16. $OD_{414}$ values from the IELISAs were compared to those obtained with the strong control serum included in each 96-well plate and a relative percent positivity value (%P) was calculated as follows (Nielsen *et al.*, 2004): $\%P=(OD_{414}$ of test sample/$OD_{414}$ of strong control serum) $\times 100$.

Diagnostic specificity and sensitivity were determined initially with 95 % confidence limits by plotting the data for negative and positive samples on a frequency histogram. Data were subsequently analysed by receiver-operator characteristics analysis (Schoonjans *et al.*, 1995).

## RESULTS AND DISCUSSION

We used RSAT as a screening test to study patients with symptoms and/or epidemiology compatible with brucellosis but negative results to tests with smooth-*B. abortus* antigen. Another objective was to ascertain the usefulness of an IELISA as a confirmatory test and to determine the cut-off value.

The serological study was run on 179 sera. One hundred and ten sera from healthy people were examined and found to be negative by tests using smooth *B. abortus* antigen (BPA, TAT, CF and CELISA) and RSAT (*B. canis* M− antigen), and when

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tested with IELISA showed a mean %P value of 16 and a standard deviation (SD) of 5·25. Fig. 1 shows the frequency distribution of these sera. Therefore a cut-off value of 26·5 %P (mean+2 SD) was established and then adjusted to 27 %P by receiver-operator characteristic curve using both positive and negative serum samples, resulting in 100 % sensitivity and specificity.

Table 1 shows the serological test results for the 17 sera from patients with positive *B. canis* culture or with close contact with culture-positive dogs, at the time that they came to our laboratory. IELISA sensitivity was 100 % with a cut-off value of 27 %P. Most of these patients presented clinical symptoms similar to brucellosis caused by other species of *Brucella* such as fever, asthenia and hepatosplenomegaly. Some of them received antibiotic treatment and made good progress. The serological follow-up performed on serial serum samples from patients who received treatment showed that the RSAT and IELISA tests correlated well with clinical progress. The boy aged 13, who spent 2 weeks in hospital with intermittent fever and hepatosplenomegaly as prominent signs, recovered normally without treatment and was clinically symptom-free 3 months afterwards, with RSAT and IELISA titres declining slowly. Two dogs in contact with the patient were serologically examined, and both gave RSAT-positive results, though only the female (which had four times given birth to weak pups that subsequently died) was IELISA positive.

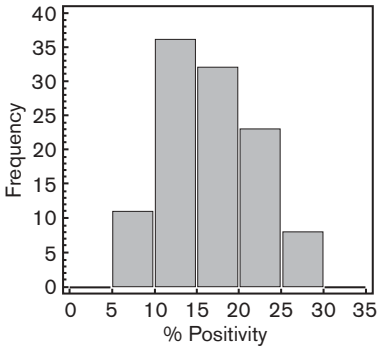


**Fig. 1.** Frequency distribution of IELISA results from 110 serum samples negative for antibodies to *B. canis*. The cut-off value of 27 %P was selected to provide an assay specificity value of 100 %.

In the group of 35 patients suspected of having brucellosis (with negative blood culture but positive RSAT), of the 25 that came to our laboratory only once, 13 were positive by IELISA, with RSAT titres that ranged from weak reaction with pure serum to dilution 1 : 4 (data not shown). Of the eight patients that came twice, three were IELISA-positive, with RSAT titres ranging from weak reaction with pure serum to dilution 1 : 32. The two patients that came to the laboratory four and five times were RSAT- and IELISA-positive. The 18 RSAT and IELISA-positive cases were from 12 men (age range, 26–61) and six women (age range, 23–

**Table 1.** Serological response of sera from patients *B. canis* culture-positive or with close contact with *B. canis*-culture-positive dogs

*B. canis* culture-positive dogs were those that were BPA-, TAT-, CF- and CELISA-negative.

| Patient | Age* (years) | RSAT† | IELISA (%P)‡ | Epidemiological data |
|---|---|---|---|---|
| 1 | 13 | 4 | 100 | *B. canis*-positive dog owner |
| 2 | 31 | 16 | 64 | *B. canis* culture-positive |
| 3 | 26 | Pos+/-§ | 36 | *B. canis*-positive dog handler |
| 4 | 39 | Pos+/-§ | 59 | *B. canis*-positive dog owner |
| 5 | 16 | 32 | 96 | *B. canis* culture-positive |
| 6 | 31 | Neg | 59 | *B. canis*-positive dog owner |
| 7 | 58 | Pos | 29 | *B. canis*-positive dog owner |
| 8 | 10 | Neg | 28 | *B. canis*-positive dog owner |
| 9 | ND | 32 | 100 | *B. canis*-positive dog handler |
| 10 | 30 | Neg | 41 | *B. canis*-positive dog owner |
| 11 | 35 | Neg | 33 | *B. canis*-positive dog owner |
| 12 | 44 | Neg | 37 | *B. canis*-positive dog owner |
| 13 | 16 | Neg | 33 | *B. canis*-positive dog owner |
| 14 | 40 | 2 | 35 | *B. canis*-positive dog owner |
| 15 | 13 | Neg | 35 | *B. canis*-positive dog owner |
| 16 | 23 | Pos | 28 | *B. canis*-positive dog owner |
| 17 | 48 | 2 | 78 | *B. canis* culture-positive |

*ND, No data.

†Reciprocal titres.

‡IELISA cut-off, 27 %P.

§Weakly positive.

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88). One was a veterinarian, two were owners of infected dogs, 10 worked with domestic animals and five presented ambiguous epidemiological information. Of the 17 IELISA-negative sera, two were RSAT-positive at dilution 1 : 2 and 15 were weakly positive with pure serum; with 2ME-RSAT, 11 tested negative and six tested weakly positive (data not shown). These sera were probably from patients at an early stage of the infection or indicated false-positive results.

Cross-reactions between *Brucella* species and other micro-organisms that share antigenic determinants causing false-positive reactions have been reported (Corbel, 1985). Similarities in the O-polysaccharide chemical structure of various micro-organisms, such as *Escherichia coli* O157 : H7, *Francisella tularensis*, *Vibrio cholerae*, *Salmonella* group N and *Pseudomonas maltophilia*, are responsible for most observed cross-reactions (Nielsen *et al.*, 2004). But no cross-reaction was observed to RSAT or to IELISA in the nine sera studied from patients with infectious diseases other than brucellosis (two with meningitis, two with haemolytic uraemic syndrome, two with salmonellosis, two with diarrhoeas and one with bloody diarrhoea), as shown in Table 2.

However, cross-reactivity was evident in sera from eight patients who had positive haemocultures of *B. abortus* biovar 1, *Brucella melitensis* biovar 1 or *Brucella suis* biovar 1. This shows that RSAT and IELISA antigens prepared with *B. canis* M− strain have LPS determinants specific for *B. canis* as well as other antigenic components shared with rough- and smooth-*Brucella* strains. Cases 1 and 2 (Table 3) were positive to both tests 17 and 45 months after admission, respectively, while case 3, from whom *B. abortus* biovar 2 was isolated, tested RSAT- and IELISA-negative at admission and 2 months later. Case 4 was negative 3 months after admission, and case 5 continued to be positive 5 months later, while the last three cases with *B. suis* haemocultures presented high titres by RSAT and IELISA. Case 8 was a dog breeder whose dogs presented clinical symptoms of brucellosis and were serology-positive with smooth-*Brucella* antigen and negative with rough-*Brucella* antigens, but the dogs' haemocultures were negative, probably because they had received antibiotic therapy.

Although *B. canis* is recognized as the aetiological agent of an infection in humans and dogs, available information on its prevalence is limited. Several serological surveys have been performed using the TAT in selected population groups: a

**Table 2**. Serological response of sera from patients with diagnoses other than brucellosis

| Patient | Age* | RSAT | IELISA (%P)† | Diagnosis | Strain isolated |
|---|---|---|---|---|---|
| 1 | 53 y | Neg | 23 | Meningitis | *Neisseria meningitidis* |
| 2 | 56 y | Neg | 22 | Meningitis | *N. meningitidis* |
| 3 | 10 m | Neg | 13 | Haemolytic uraemic syndrome | *E. coli* O157 |
| 4 | ND | Neg | 18 | Bloody diarrhoea | *E. coli* O157 |
| 5 | ND | Neg | 19 | Haemolytic uraemic syndrome | *E. coli* O157 |
| 6 | 2 y | Neg | 13 | Diarrhoea | *E. coli* O157 |
| 7 | 2 y 9 m | Neg | 9 | Diarrhoea | *E. coli* O157 |
| 8 | 40 y | Neg | 21 | Salmonellosis (Widal positive H : 160 O : 80) | |
| 9 | ND | Neg | 26 | Salmonellosis (Widal positive H : 80 O : 360) | |

*ND, No data; m, months; y, years.

†IELISA cut-off, 27 %P.

**Table 3.** Serological response of sera from patients with brucellosis caused by smooth *Brucella* species

| Patient | BPA | TAT* | CF* | CELISA (%I)† | RSAT* | IELISA (%P)‡ | Species/biovar |
|---|---|---|---|---|---|---|---|
| 1 | Pos | 1600 | 320 | 77 | 512 | 96 | *B. abortus* 1 |
| 2 | Pos | 400 | 80 | 67 | 64 | 100 | *B. abortus* 1 |
| 3 | Pos | 1600 | 40 | 65 | Neg | 25 | *B. abortus* 2 |
| 4 | Pos | 800 | 40 | 52 | Pos | 49 | *B. melitensis* 1 |
| 5 | Pos | 800 | 320 | 74 | Pos | 90 | *B. melitensis* 1 |
| 6 | Pos | 6400 | 640 | 69 | 64 | 100 | *B. suis* 1 |
| 7 | Pos | 400 | 640 | 83 | 32 | 100 | *B. suis* 1 |
| 8 | Pos | 400 | 160 | 93 | 4 | 96 | *B. suis* 1 |

*Reciprocal titres.

†CELISA cut-off, 28 %I.

‡IELISA cut-off, 27 %P.

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study of hospital patients with various complaints in Mexico revealed a 13 % prevalence of significant antibody titres (Flores-Castro & Segura, 1976); in US military populations 0·4 % positive reactions were detected (Lewis & Anderson, 1973); 0·59 % in Florida residents (Hoff & Nichols, 1974; Hoff & Schneider, 1975) and 67·8 % in Oklahoma (Monroe *et al.*, 1975); an investigation in Germany found antibody titres in 6 out of 1915 sera (Carmichael *et al.*, 1980). Another study found 21 out of 1065 people to be positive to *B. canis* antibodies by the gel-diffusion test using *B. ovis* antigen (Varela-Diaz & Myers, 1979) and a microagglutination test using a safranin-dyed *B. canis* antigen detected the infection in four patients with febrile illness (Polt & Schaefer, 1982). For TATs using rough-*Brucella* antigens, the difficulty of establishing a cut-off point and the significant agglutinin titres from non-specific reactions has been recognized (Carmichael *et al.*, 1980).

Understanding that patients showing symptoms compatible with brucellosis could potentially be infected by *B. canis*, we recommend the use of RSAT and IELISA tests to check sera from cases with negative serological tests to smooth-*Brucella* antigen.

It is generally agreed that available evidence suggests a low incidence of clinical and subclinical human brucellosis due to *B. canis*. But it has been emphasized that routine brucellosis diagnosis does not include *B. canis* investigation so infection with this *Brucella* species may be more widespread than is now suspected. After studying a larger number of samples the RSAT could provide a suitable screening test for the diagnosis of *B. canis* human brucellosis, while a supplementary technique such as IELISA performed on all positive RSAT samples could ensure diagnostic specificity and confirm the diagnosis.

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