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Protean clinical manifestations and diagnostic challenges of human brucellosis in adults: 16 years' experience in an endemic area

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A prospective study was carried out to elucidate the clinical, epidemiological and laboratory features of human brucellosis. A total of 26 948 blood samples (from adults aged 15 years and above) were screened for serological evidence of brucellosis over a period of 16 years. The slide agglutination/Rose Bengal plate agglutination test gave positive results in 517 patients, of which 509 had detectable titres by the standard tube agglutination test (SAT). The diagnosis of brucellosis was documented in 495 (1·8 %) patients based on diagnostic titres ($\geqslant$1 : 160, 490 cases) and rising titres from insignificant titres (four cases) by serology and for one case by blood-culture isolation alone. Blood cultures were carried out in 345 cases, of which 191 cases (55·3 %) yielded *Brucella melitensis*. In 77/79 cases undertaken for follow up, there was a steady fall in 2-mercaptoethanol (2ME) agglutination titres along with clinical improvement ($P < 0{\cdot}01$). SAT titres remained detectable in most cases for a longer period in spite of an effective antimicrobial therapy and clinical recovery. A substantial number of patients (84·2 %) presented with fever, this being the only complaint in 51·1 % of the cases. Complications were present in 8·8 % of the patients (arthritis excluded): this included the unusual complications of hydrocele (two cases), Stevens–Johnson syndrome (one case) and urinary tract infection (one case). *Brucella* agglutinins were demonstrated in synovial, testicular, hydrocele and cerebrospinal fluids. There was no clinical suspicion of brucellosis in 439 cases (88·7 %) and the diagnosis was made only by routine serology. A two-drug regimen for 42–84 days with a follow-up 2ME test resulted in lower levels of relapse. These results suggest that, in endemic areas of the world, it should be mandatory to screen routinely for brucellosis due to protean clinical manifestations.

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INTRODUCTION

Brucellosis is a worldwide zoonotic disease caused by *Brucella* spp. The genus *Brucella* comprises Gram-negative, facultative, intracellular pathogens (Alton *et al.*, 1975). Currently, there are six recognized species of *Brucella* based on phenotypic characteristics, antigenic variation and prevalence of infection in different animal hosts: *Brucella abortus* (cattle), *Brucella canis* (dogs), *Brucella melitensis* (goats, sheep), *Brucella neotomae* (desert wood rats), *Brucella ovis* (sheep) and *Brucella suis* (pigs, reindeer and hares) (Corbel, 1997; Moreno *et al.*, 2002). Recently, two *Brucella* strains from marine mammals have been reported (Bricker *et al.*, 2000; Cloeckaert *et al.*, 2000) and the names *Brucella pinnipediae* (seal/otter) and *Brucella cetaceae* (porpoise/whale) have been proposed (Cloeckaert *et al.*, 2003). There has also been a report of human infection with marine brucellae (Sohn *et al.*, 2003). Although each species of *Brucella* has a preferred host, all can infect a wide range of animals, including humans. Brucellosis is a worldwide re-emerging zoonosis causing high economic losses and severe human disease. It has areas of high endemicity such as the Mediterranean, the Middle East, Latin America and Asia (Corbel, 1997). Brucellosis remains an uncontrolled public health problem worldwide. In many developing countries, the problem is compounded by the absence of national surveillance programmes, diagnostic facilities and reliable data (Cooper, 1992).

Human infection can occur through consumption of infected raw milk, raw milk products and raw meat. Other means of infection include skin abrasions and inhalation of airborne animal manure particles. In addition, occupational

Abbreviations: CSF, cerebrospinal fluid; 2ME, 2-mercaptoethanol; RBPT, Rose Bengal plate agglutination test; SAT, standard tube agglutination test.

exposure of abattoir workers, veterinarians and laboratory technicians can result in transmission of the disease through contaminated aerosols. Transmission of *B. melitensis* from person to person has also been reported in the literature (Mantur *et al.*, 1996; Wyatt, 1996). Brucellosis may present clinically as acute, as chronic following an acute attack or as chronic and of insidious onset. Human brucellosis is a multisystem disease that may present with a broad spectrum of clinical manifestations and its complications can affect almost all organs and systems with varying incidence (Andres Morist *et al.*, 2003; Cesur *et al.*, 2003; Gur *et al.*, 2003). Its clinical manifestations and focal complications are often troublesome in making a clinical diagnosis. Its diagnosis therefore requires microbiological confirmation by means of isolation from blood culture or demonstration of the presence of specific antibodies by serological tests. The diagnosis of brucellosis based exclusively on *Brucella* isolation presents several drawbacks. The slow growth of *Brucella* in primary cultures may delay diagnosis for more than 7 days (Yagupsky, 1999). Also, blood-culture sensitivity is often low, ranging from 50 to 90 % depending on the disease stage, *Brucella* species, culture medium, quantity of circulating bacteria and the blood-culture technique employed (Mantur & Mangalgi, 2004; Yagupsky, 1999). Hence, serological tests play a major role in cases when the disease cannot be detected by blood culture. However, the interpretation of these tests is often difficult, particularly in patients with chronic brucellosis, in reinfections and relapses, and in areas of endemicity where a high proportion of the population has antibodies against brucellosis. Debate continues regarding the best antibiotic combination for the treatment of human brucellosis (Bayindir *et al.*, 2003; El Miedany *et al.*, 2003).

This paper analyses our experiences of the past 16 years with human brucellosis in adults presenting with protean clinical manifestations and the diagnostic challenges faced in an endemic area. The serological screening method was employed for initial case identification of brucellosis in this endemic area. Positive cases detected by this screening method were then subjected to standard tube agglutination and 2-mercaptoethanol (2ME) agglutination tests for interpretation and to establish the diagnosis of brucellosis in this area.

METHODS

***Brucella* antibody demonstration.** A total of 26 948 serum samples submitted to the Microbiology Laboratory, BLDEA's Shri B. M. Patil Medical College and Hospital, Bijapur, Karnataka, India, over a period of 16 years spanning August 1988 to August 2004 were screened for evidence of brucellosis. Screening was done by slide agglutination or Rose Bengal plate agglutination test (RBPT) (Alton *et al.*, 1975). If the screening test result was positive for antibodies (517 patients), the clinician was informed and after obtaining informed consent, a standard tube agglutination test (SAT) was carried out (Alton *et al.*, 1975) and clinical and epidemiological data were recorded. *B. abortus* antigens were supplied by the Indian Veterinary Research Institute (IVRI), Izatnagar, India.

For nine cases of epididymo-orchitis (eight seropositive, one seronegative), clear aspirate obtained from the testes was used instead of serum in the above tests using the same procedure. In addition, semen samples were obtained from eight patients: one drop of a 1 % solution of sodium azide was added to 1 ml semen and left for 30 min. The samples were centrifuged at 75 ***g*** (R4C; Remi) for 10 min and the supernatant fluid was used instead of serum in the above-mentioned tests (Brinley Morgon *et al.*, 1978).

The following samples from seropositive patients were also tested for evidence of brucellosis using the above techniques: 32 knee joint fluid samples, two ankle joint fluid samples, seven cerebrospinal fluid (CSF) samples and two hydrocele fluid samples.

2ME agglutination test. All of the 517 sera found to be positive by the screening test were tested in parallel with the 2ME agglutination test. 2ME agglutination was performed with *B. abortus* plain antigen, except that 2ME was added to each tube to a final concentration of 0·05 M (Buchanan *et al.*, 1974).

Paired sera available from 217 patients were tested for SAT and 2ME titres. Multiple sera were also tested during follow up for both SAT and 2ME titres in 79 cases; the follow-up period ranged from 14 to 720 days with a median follow up of 106 days.

In addition to the above, 102 family members of 18 indexed, bacteriologically confirmed cases were also investigated for evidence of brucellosis.

Isolation and identification of *Brucella*. Blood cultures were performed from 345 cases, which included 15 patients who had insignificant SAT titres of 1 : 20 and one patient with seronegative epididymo-orchitis due to clinical/epidemiological indication. Venous blood (5 ml) was inoculated aseptically into the broth phase of Castaneda's biphasic medium containing brain–heart infusion agar and broth or trypticase soy agar and broth (High Media) in duplicate. The media were incubated at 37 °C with and without a CO_2 atmosphere and examined for bacterial growth once a day for 30 days, tilting the broth/blood mixtures over the solid phase every day.

A number of other samples (nine bone marrow, nine testicular fluid, 34 synovial fluid, eight semen, seven CSF, two hydrocele fluid and two ascitic fluid) were also cultured using Castaneda's biphasic technique as described above.

Identification of *Brucella* strains was done using standard classification tests, including Gram staining, a modified Ziehl–Neelsen stain, growth characteristics, oxidase activity, urease activity, H_2S production (4 days), dye tolerance such as basic fuchsin (1 : 50 000 and 1 : 100 000) and thionin (1 : 25 000, 1 : 50 000 and 1 : 100 000) and seroagglutination. *B. abortus* and *B. melitensis* monospecific antisera (Murex Biotech) were used for seroagglutination tests. Isolates were sent to IVRI, Izatnagar, India, for confirmatory identification.

Statistics. To determine the significance of the difference between the performance of the two agglutination tests (SAT and 2ME) and the level of antibodies at different stages of follow up, an analysis of variance was applied. A value of $P < 0{\cdot}05$ was considered significant.

RESULTS AND DISCUSSION

The slide agglutination/Rose Bengal plate agglutination test was positive in 517 subjects (1·9 %). Patients who gave a positive screening result were retested by the SAT from which 509 cases showed titres ranging from 1 : 20 to 1 : 10 240 with a geometric mean titre $\pm$ SD of 1 : 288·3 $\pm$ 1423·2.

The diagnosis of brucellosis was established in 495 patients (1·8 %) based on diagnostic titres ($\geqslant$1 : 160, 490 cases) and rising titres from insignificant titres (four cases) by serology and for one case on blood-culture isolation alone (seronegative epididymo-orchitis). The data clearly elucidate the endemicity of brucellosis in this area as shown by the occurrence of cases throughout the year, although a small peak was observed in the months of July and August. Calving of farm animals usually occurs in April and May in this area, which could explain the peak incidence of human brucellosis seen in July and August in this study. The reported incidence and prevalence of the disease vary widely from country to country. Karabay *et al.* (2004) have reported a low prevalence of 1 % in certain areas of Turkey, which is lower than the prevalence reported here, although another study from Turkey (Sumer *et al.*, 2003) and one from Saudi Arabia (Al-Sekait, 1999) have reported higher prevalences of 3·2 and 4·5 %, respectively. This difference in endemicity may be due to the prevalent practices in the population and also the incidence of brucellosis in the community. We were able to detect an additional seven cases of brucellosis, five of which were bacteraemic, from household members of 18 indexed, bacteriologically confirmed cases. Symptomatic infection among other family members was quite common. Almuneef *et al.* (2004) have also reported similar findings. This finding reflects the value of screening family members of index cases of acute brucellosis in an endemic area to pick up additional unrecognized cases. This must be taken into account by the physicians caring for these patients, so that timely diagnosis and provision of therapy occur, resulting in lower morbidity.

Four cases with non-diagnostic SAT titres of 1 : 20 revealed rising titres in paired sera from the four cases, along with positive blood cultures in two. Our data clearly indicate that SAT titres of $<$1 : 160 cannot always be disregarded without follow up. Conversely, SAT titres of $\geqslant$1 : 160 do not always signify active infection, especially in *Brucella*-endemic areas. The SAT measures the total amount of agglutinating antibodies (IgM and IgG), whilst the 2ME agglutination test measures IgG antibodies only. This differentiation is important, as IgG antibodies are considered a better indicator of active infection than IgM and the rapid fall in the level of IgG antibodies is said to be prognostic of successful therapy (Buchanan & Faber, 1980). A survey conducted by Almuneef & Memish (2002) in Saudi Arabia found various levels of *Brucella* SAT antibodies in many clinically cured patients. Seventy-nine patients diagnosed as having active brucellosis in the present study were followed up for different lengths of time (median follow up of 106 days) and monitored for *Brucella* antibodies by SAT and 2ME agglutination. In most cases, *Brucella* SAT titres remained measurable, in spite of falling to low levels (Fig. 1) ranging from 1 : 160 to 1 : 640 (significant titres), despite an effective therapy and clinical cure. This emphasizes the overdiagnosis and diagnostic challenges faced in an area where typhoid, malaria, tuberculosis and rheumatoid arthritis clinically mimic human brucellosis, thereby exposing/denying patients access to specific therapy. However, a remarkable finding of our study was that there was a sustained drop in 2ME titres in 97·5 % of cases (Fig. 1, P $<$0·01), reflecting the importance of the 2ME test for diagnosis of brucellosis in conjunction with the SAT, as well as for follow up of brucellosis in *Brucella*-endemic countries. Gazapo *et al.* (1989) claimed that ELISA was an excellent method for follow up of brucellosis; however, the results of our study clearly indicate that the 2ME agglutination test is a useful assay, as it is inexpensive and technologically simple with stable reagents. All 494 patients had an active infection, as determined by the presence of 2ME titres (IgG) ranging from 1 : 20 to 1 : 5120 with a geometric mean titre $\pm$ SD of 1 : 131 $\pm$ 641. The disease is notorious for its relapses (Bayindir *et al.*, 2003; El Miedany *et al.*, 2003). In the present series, only two patients relapsed. Extending treatment for longer than previously recommended (6 weeks) results in an incidence of relapse significantly lower than for shorter courses of treatment. No relapse was seen for up to 6 months of follow up in all of the patients who completed therapy for 6 weeks in a study by Gaafar (1997). The median duration of therapy and follow up of patients in the present study was 49 and 106 days, respectively. Another important finding of this study was seronegative (RBPT, SAT, 2ME test, Coombs test) brucellar epididymo-orchitis reported in one patient. Patients with acute or subacute brucellosis may, on rare occasions, fail to mount a humoral-mediated immune response. In the literature, however, there are reports of cases of brucellosis associated with seronegativity (Potasman *et al.*, 1991; Yavuj *et al.*, 2004). It appears

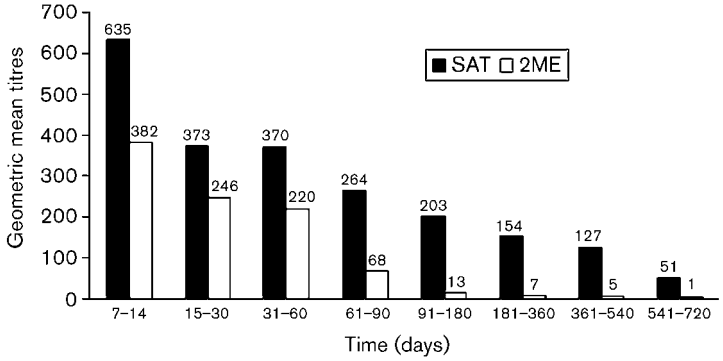

Fig. 1. Results of the SAT and 2ME tests at different follow-up times in 79 cases. In most cases, in spite of falling to low levels, *Brucella* SAT titres remained measurable with significant titres despite an effective therapy and clinical cure, but there was a sustained drop in 2ME titres in 97·5 % (77/79) of cases.

clear that seronegativity may be a possibility in cases of brucellosis presenting with atypical lesions. However, our case was unique in that *Brucella* agglutinins were detected by the RBPT and SAT in the testicular fluid. Prozone, a commonly reported phenomenon in brucellosis (Young, 1983), was seen in only three cases in the present study up to a dilution of 1 : 640.

Blood cultures were available from 345 cases, of which 191 (55·3 %) specimens yielded *B. melitensis*. This also included two cases with an initial SAT titre of 1 : 20 and one patient with seronegative epididymo-orchitis. Of these cases, 188 were biotype 1 and three were biotype 3. This is similar to isolations in other studies carried out elsewhere in the world (Gotuzzo *et al.*, 1986; Namiduru *et al.*, 2003). Of nine simultaneous bone marrow cultures done, seven (77·7 %) grew *B. melitensis* biotype 1. Of the seven, two specimens were positive only by bone marrow cultures. Because of the suboptimal recovery rate of brucellae from blood, it has been suggested that cultures of bone marrow may improve the recovery rate of the organism although the procedure is painful. Our findings are consistent with those of Gotuzzo *et al.* (1986). A significant finding in the present series was the rapid urease production ($<$30 min) seen in 96·8 % of our strains. Therefore, we suggest that *B. melitensis* may be classified as a rapid urease producer along with *B. suis* and *B. canis*. This finding could be exploited in the rapid identification of *Brucella* isolates to the genus level. This characteristic could also be used for rapid growth identification of brucellae from clinical specimens using urea broth in areas where these rapid urease-producing *Brucella* strains have been reported. This latter suggestion requires further investigation. The modified Ziehl–Neelsen stain employed in this study for culture identification is usually used for demonstration of *Brucella* in clinical specimens for the diagnosis of animal brucellosis (Alton *et al.*, 1975). We routinely use this staining method following confirmation of the strain by Gram staining, and obtained positive results in all of the strains tested here. We strongly recommend Gram-stain morphology and modified ZN staining, coupled with the urease test for rapid identification of *Brucella* to the level of genus where facilities for further identification are not available. This would facilitate the early institution of appropriate antimicrobial therapy, thereby reducing morbidity.

There was a predominance of males in this study. Human brucellosis affects all age groups (Sauret & Vilissova, 2002). Men between the ages of 15 and 86 years accounted for 390 of the cases, with a mean age of 31 years. The most common ages involved in this study were adolescent and young adults, as has been described in other studies (Gaafar, 1997; Madkour *et al.*, 1985). This reflects the magnitude of the socio-economic impact of brucellosis in this area, as it affects mainly the most productive group in the community. We found that sexual intercourse was implicated as a possible means of transmission in six cases, in addition to the well-established modes of transmission.

As shown in Table 1, the overall clinical picture of brucellosis in our study was very similar to that reported by workers elsewhere in the world (Barroso *et al.*, 2002; Gaafar, 1997; Namiduru *et al.*, 2003). In 88·7 % of cases (Table 2), there was no clinical suspicion of brucellosis and in 1·2 % of cases, brucellosis was placed as a differential diagnosis in either second or third place. The illness was acute in 310 cases (62·6 %), subacute in 146 (29·4 %) and 39 (7·8 %) had chronic brucellosis. A substantial number of patients (84·2 %) presented with fever, with fever being the only complaint in 51·1 % of the cases. Joint pain alone was

Table 1. Clinical findings in 495 patients infected with *B. melitensis*

Symptoms/signs	No. of patients (%)
Fever ($>$37·5 °C)	417 (84·2)
Joint pain	117 (23·6)
Low backache	89 (17·9)
Night sweats	19 (3·8)
Cough, breathlessness, haemoptysis	18 (3·6)
Testicular pain, scrotal swelling, burning micturition	14 (2·8)
Pain in abdomen, nausea, vomiting, jaundice	13 (2·6)
Headache	8 (1·6)
Fatigue	6 (1·2)
Papules*, mouth ulcers	5 (1)
Convulsions	1 (0·2)
Splenomegaly	95 (19·2)
Hepatomegaly	56 (11·3)
Hepatosplenomegaly	49 (9·8)
Lymphadenopathy	12 (2·4)

*One case was also associated with subcutaneous nodules.

Table 2. Clinical diagnosis of 495 cases following initial examination

Principal/differential diagnosis	No. of cases (%)
Enteric fever	196 (39·6)
Malaria	102 (20·6)
Arthritis	67 (13·5)
Brucellosis	50 (10·1)
Pyrexia of unknown origin	26 (5·2)
Epididymo-orchitis, bilateral hydrocele, urinary tract infection	14 (2·8)
Tuberculosis	8 (1·6)
Chronic liver disease, splenic abscess, acute cholecystitis	7 (1·4)
Endocarditis	6 (1·2)
Bronchitis, pneumonia	5 (1)
Skin rashes, Stevens–Johnson syndrome	5 (1)
Meningitis	4 (0·8)
Human immunodeficiency virus infection	3 (0·6)
Encephalitis	3 (0·6)
Malaria, enteric fever, brucellosis*	2 (0·4)
Enteric fever, brucellosis*	2 (0·4)
Pulmonary tuberculosis, brucellosis*	1 (0·2)
Rheumatic arthritis, brucellosis*	1 (0·2)

*Differential diagnosis.

found in 29 cases, and in 22 cases low backache alone was recorded. The clinical features in human brucellosis are protean and therefore a number of infectious and non-infectious diseases may mimic brucellosis clinically. Medical literature reports underdiagnosis of brucellosis cases, which was clearly shown in our study where 88·7 % of cases would have been missed if routine serological surveillance had not been done. Routine serological surveillance is not practised even in *Brucella*-endemic areas, and we suggest that this should be a part of laboratory testing coupled with a high index of clinical suspicion to improve the level of case detection. In our study, *B. melitensis* was recovered from the synovial fluid of 21/34 (61·7 %) brucellar arthritis patients. This is similar to another study (Yagupsky & Peled, 2002). It is worth noting that *Brucella* agglutinins were demonstrated in the synovial fluid of all 34 patients in the present study. There is no report of the demonstration of *Brucella* agglutinins in joint fluid in the literature. Seropositivity for human immunodeficiency virus was also found in 17 brucellosis patients. There was no diagnostic/therapeutic difficulty, as has been noted in the literature (Moreno *et al.*, 1998; Paul *et al.*, 1995; Pedro-Botet *et al.*, 1992).

Table 3 shows the complications that were encountered in 44/495 (8·8 %) patients. To our knowledge, *Brucella*-specific antibodies have not been demonstrated previously in the testicular fluid of epididymo-orchitis patients. In the present study, *Brucella* agglutinins were demonstrated in the testicular fluid of eight patients along with antibodies in the semen of five patients and we were also successful in recovering *B. melitensis* from the testicular fluid of seven out of nine patients; the semen was sterile in the eight patients studied. All seven patients with neurobrucellosis involving the meninges had positive agglutinin titres in CSF with none yielding cultures. In the present study, five cases of chronic liver disease due to *B. melitensis* infection were reported. Spontaneous bacterial peritonitis was seen as a complication in two cases and the diagnosis was established by obtaining *B. melitensis* from ascitic fluid cultures.

Table 3. Complications of brucellosis

Complication	No. of cases
Genitourinary tract	
Epididymo-orchitis	11
Hydrocele	2
Urinary tract infection	1
Neurobrucellosis	
Meningitis	4
Meningoencephalitis	3
Endocarditis	6
Cutaneous/mucous membrane lesions*	5
Gastrointestinal tract	
Chronic liver disease	5
Splenic abscess	1
Ac. cholecystitis	1
Respiratory system	
Pneumonia	2
Bronchitis	3
Total	**44**

*Included a case of Stevens–Johnson syndrome.

We report here for the first time unusual presentations that were noted in four patients, two with hydrocele (bilateral), one with Stevens–Johnson syndrome and one with urinary tract infection. The diagnosis of hydrocele of *Brucella* origin was confirmed by detecting *Brucella* agglutinins in hydrocele fluid of both patients, with recovery of *B. melitensis* from one patient. Both patients responded well to medical therapy alone, receiving a 56 day course of antimicrobial therapy. One case had painful ulcerative lesions in the oral cavity with fever and joint pain and was clinically diagnosed as Stevens–Johnson syndrome. SAT and 2ME titres in this patient were 1 : 10 240 and 1 : 640, respectively. One case had a 3 month history of fever and burning micturition with enlargement of the inguinal lymph nodes and was diagnosed as a urinary tract infection. The blood culture was positive for *B. melitensis* and *Brucella* agglutinins were found with titres of 1 : 320 and 1 : 160 by the SAT and 2ME test, respectively. However, urine culture was sterile for *Brucella* and other bacteria. Although many organ systems may be involved, brucellosis is rarely fatal (Al Dahouk *et al.*, 2003). No mortality was seen in the present series.

All 495 patients received a standard regimen of two drugs consisting of tetracycline orally (2 g per day in four divided doses) for 42–84 days together with streptomycin (0·75–1 g per day intramuscularly) for the first 14–28 days depending on resolution of signs and symptoms. Rifampicin (450–600 mg per day orally) was also added for the treatment of meningitis and meningoencephalitis. Rifampicin (600 mg per day orally) was added to the above two-drug regimen extending therapy for a further 84 days to treat the relapsed cases and the outcome was remarkable.

In conclusion, laboratory surveillance in endemic areas is essential for the diagnosis and effective treatment of protean human brucellosis. Development of new specific and cost-effective diagnostic algorithms for developing countries needs to be carried out, along with rapid and sensitive laboratory tests including molecular methods, to decrease morbidity in the endemic population. Although PCR is promising, standardization of the extraction methods and the set up is lacking and a better understanding of the clinical significance of the results is still needed (Navarro *et al.*, 2004). The use of molecular methods in endemic areas needs to be explored before they can be used in these areas to diagnose brucellosis.

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