Cloning and expression of the immunoreactive *Brucella melitensis* 28 kDa outer-membrane protein (Omp28) encoding gene and evaluation of the potential of Omp28 for clinical diagnosis of brucellosis

Duraipandian Thavaselvam, Ashu Kumar, Sapana Tiwari, Manvi Mishra and Archana Prakash

Correspondence

Duraipandian Thavaselvam
dtselvam@drde.drdo.in or
dtselvam@rediffmail.com

Division of Microbiology, Defence Research & Development Establishment, Jhansi Road, Gwalior 474 002, India

Brucellosis is a disease caused by Gram-negative, facultative, intracellular bacteria belonging to the genus *Brucella*. It is an emerging zoonosis, and an economically important infection of humans and livestock with a worldwide distribution. Human infection is known to occur through consumption of infected raw milk, milk products and undercooked or raw meat. Serodiagnosis of brucellosis is carried out by detection of antibodies generated against LPS or whole-cell bacterial extracts by ELISA or agglutination tests using colorimetry. The present study was designed to develop a highly sensitive and specific indirect ELISA in both a microtitre plate and dot-blot format employing the recombinant outer-membrane protein 28 (rOmp28). Cloning and expression of *Brucella melitensis* Omp28 protein, which is a group 3 antigen, was accomplished by PCR amplification and cloning of the gene in a pET-28a expression system, followed by Ni-NTA affinity chromatography purification of the His-tagged recombinant protein. An indirect ELISA in both a microtitre plate and dot-blot format was optimized with sera collected from three groups: culture-confirmed cases, clinically suspected cases and healthy individuals. The rOmp28 protein reacted only with the culture-confirmed positive samples and no reaction was observed with culture-negative samples, confirming the immunoreactivity of the recombinant protein. The test in both formats had a correlation of approximately 90 % with the Rose Bengal plate agglutination test (RBPT) and a standard tube agglutination test, assays that are routinely performed for the serodiagnosis of brucellosis. The sensitivity and specificity of the assay in the plate format were 97.50 and 85.59 %, and in the dot-blot format were 82.05 and 92.43 %, respectively, in comparison with RBPT. The specificity of this assay was further confirmed by testing samples that were positive for malaria and typhoid, which gave negative results. This ELISA system in microtitre plates and a dot-blot format will be useful for the rapid screening of large numbers of samples for the diagnosis of human brucellosis in endemic areas.

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INTRODUCTION

Brucellosis is a major veterinary and human public-health problem in rural tropical communities in most parts of the world. Human infections with *Brucella melitensis* are common (Corbel, 1997; Moreno *et al.*, 2002), and the incidence of brucellosis in livestock is of great economic concern due to reduced productivity, increased numbers of

abortions and weak offspring, and is a major impediment to trade and export of livestock. Human brucellosis is a severe debilitating disease that requires prolonged treatment with the use of several antibiotics, and also involves considerable medical expense, as well as loss of working hours. The main mode of transmission of this disease to humans is through the consumption of untreated milk and milk products. This disease remains an uncontrolled problem, mostly in highly endemic regions such as the Mediterranean, Middle East, Africa, Latin America and parts of Asia, including India (Lopez, 1989; Corbel, 1997; Refai, 2002). Worldwide, it is estimated that a few million

Abbreviations: DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase; OMP, outer-membrane protein; RBPT, Rose Bengal plate agglutination test; rOmp, recombinant outer-membrane protein; STAT, standard tube agglutination test.

cases of human brucellosis occur per year, but no accurate estimate on the prevalence of brucellosis is available due to the lack of facilities for diagnosis and reporting (WHO, 1997).

The conventional diagnosis is microbiological confirmation by means of isolation of bacteria from blood or other body fluids. The isolation rate of *Brucella* is poor due to its slow growth rate, the low quantity of circulating viable bacteria, and problems with standardization of the culture medium and blood culture techniques employed, as well as the presence of antibiotics that inhibit growth (Yagupsky, 1999). Recently, a higher rate of positive blood cultures (91 % in acute brucellosis and 74 % in chronic brucellosis) has been achieved using an improved lysis centrifugation technique (Mantur & Mangalgi, 2004). The demonstration of antibodies generated against *Brucella* using serological tests remains a viable alternative to culture, and several serological tests, such as the Rose Bengal plate agglutination test (RBPT) and a standard tube agglutination test (STAT) are the most popular serological tests used in the field for the diagnosis of brucellosis (Morgan *et al.*, 1969; Bettelheim *et al.*, 1983; Ruiz-Mesa *et al.*, 2005). The Coombs test, which detects incomplete antibodies, and immunocapture-agglutination tests have shown similar levels of performance, with higher sensitivity and specificity in the diagnosis of human brucellosis (Orduña *et al.*, 2000). Several workers have reported the development of antibody detection systems based on ELISA and lateral flow assays for the diagnosis of human brucellosis (Batra *et al.*, 1998; Gall *et al.*, 2006; Hunter *et al.*, 1986; Gad El-Rab & Kambal, 1998; Smits *et al.*, 2003). Most of the ELISAs reported are based on the cross-reactive LPS or sonicated whole-cell extracts, both of which are known to be less specific when compared with other proteins (Muñoz *et al.*, 2005). The important antigens that have been investigated in detail for the purpose of disease diagnosis are the outer-membrane proteins (OMPs) of *Brucella* species. *Brucella* OMPs are divided based on their molecular mass into group 1 antigens with a molecular mass of 94 kDa, group 2 antigens of approximately 41–43 kDa and group 3 antigens of 30 kDa. Omp28 and Omp25 are both group 3 antigens and are distinct from each other based on molecular mass (Santos *et al.*, 1984). The use of highly specific recombinant antigens, particularly the highly immunogenic OMPs, in an ELISA format offers high sensitivity and specificity in addition to a field-usable format (Jubier-Maurin *et al.*, 2001). In the present study, efforts were made to clone the gene of the highly immunogenic Omp28 protein of *B. melitensis* in the pET-28a bacterial expression system. The recombinant outer-membrane protein (rOmp28) was purified and used in an indirect microtitre plate ELISA and a dot-blot ELISA for the detection of *Brucella* antibodies in three different groups of patient serum samples: culture-confirmed cases, clinically suspected cases and healthy volunteers. Cross-reactivity was also checked by testing on serum samples positive for other fever-related illnesses. Comparisons of the ELISAs were carried out

against the agglutination-based assays RBPT and STAT, which are widely used in peripheral and secondary-level hospital care in developing countries where brucellosis is highly endemic. The correlation, sensitivity, specificity and positive and negative predictive values of the assay were calculated in comparison with RBPT and STAT. The field usability of the ELISA in a dot-blot format was also investigated for possible introduction of this ELISA as an alternative to the routinely used agglutination assays.

METHODS

Bacterial strains. The *Brucella* bacterial strains used in the study were *B. melitensis* 16M, and *Brucella abortus* S19 and S99. Strains were routinely cultured in Brucella broth (Difco Laboratories). The pET-28a vector and the host cells *Escherichia coli* BL21(DE3) were purchased from Novagen. *E. coli* was routinely grown in Lennox broth (Difco Laboratories). When these bacteria were grown in solid medium, the above medium was supplemented with 1.5 % (w/v) Bacto agar (Difco). When antibiotic selection was needed, kanamycin (Sigma) was added to the agar and broth (at 30 µg ml⁻¹).

Samples. A total of 124 patient samples collected from different hospitals and field laboratories was used in this study. In addition, 34 samples from asymptomatic healthy voluntary donors were also added to the study for comparison purposes. The sera were grouped as: group I, comprising samples that had been bacteriologically confirmed ($n=33$); group II, comprising serum samples collected from patients with suspected disease on which only serology had been carried out ($n=91$); and group III, comprising the samples collected from healthy voluntary donors ($n=34$). The six positive cultures from group I were identified as *B. melitensis* by biochemical tests and also confirmed by PCR analysis. To check for cross-reactivity, 14 samples positive for malaria (confirmed by a Giemsa-stained blood smear) and 12 samples positive for typhoid (confirmed by the Widal test) were also used in the study.

Cloning, expression and purification of recombinant Omp28 protein. Bacterial DNA from cultures of *B. melitensis* 16M grown overnight was extracted using a Qiagen DNA extraction kit. The DNA was checked using agarose gel electrophoresis, and the purity and quantity were estimated using a spectrophotometer. The region encoding the *omp28* gene, consisting of 753 bp, was amplified with the primers 5'-ACATTGGATCCATGAACACTCGTGCTAGC-3' (forward) and 5'-CGGCCAAGCTTTTACTTGATTCAAAAACGACA-3' (reverse), and ligated into pET-28a. The ligated product was then used to transform the expression host *E. coli* BL21(DE3). The exponential-phase culture of the confirmed rOmp28 clone was induced with different IPTG concentrations of 0.5, 1.0, 1.5 and 2.0 mM, and checked for expression at hourly intervals up to 5 h. Induced cells, as well as uninduced cells exposed to the same conditions, were lysed in 1 × lysis buffer and analysed by 12 % PAGE, as described by Laemmli (1970). In order to ascertain the location of the expressed recombinant protein, the bacterial cell suspension was sonicated for 10 min with a pulse interval of 8 s. The sonicated extract was centrifuged at 18 600 g for 30 min at 4 °C. The supernatant and cell pellet, with appropriate controls and molecular mass markers, were analysed by 12 % SDS-PAGE. After confirmation of the solubility, the protein was purified by His-tag binding affinity to Ni-NTA agarose. Purification of the cell lysate was carried out using a Qiagen Ni-NTA spin column with a native purification protocol as specified by the manufacturer, and bulk purification was carried out by gel filtration affinity column chromatography using Ni-NTA Superflow (Qiagen). The purified protein was checked by

SDS-PAGE followed by Coomassie blue staining, and protein concentration was estimated by the Lowry method using BSA as a standard. The purified protein was stored at temperatures of 4, 15, 25, 37 and 45 °C, and at pH values of 4, 5, 6, 7, 8 and 9 in phosphate buffer, for a period of 12, 24, 48 and 72 h. After incubation, the protein was analysed by SDS-PAGE to check its stability. After subjection to the different temperature and pH conditions, the reactivity of the protein was also further tested by ELISA.

Western blotting with positive and negative clinical samples.

The purified protein was subjected to Western blotting as described by Towbin *et al.* (1979). The electrophoretic transfer of protein from the polyacrylamide gel to a nitrocellulose membrane was carried out using Tris/glycine buffer (pH 8.3) with 20 % methanol. The protein-free sites of the nitrocellulose paper were blocked with 3 % BSA in PBS overnight at 4 °C. The blots were washed three times with PBS with 0.05 % Tween 20 (PBS-T) for 15 min and incubated with anti-His conjugate [horseradish peroxidase (HRP)-conjugated; Qiagen], washed and developed with 3,3'-diaminobenzidine (DAB)/H₂O₂. In another experiment, the membrane was also incubated with rabbit polyclonal antibodies generated against sonicated whole-cell extract of *B. melitensis* 16 M, washed and incubated with goat anti-rabbit HRP-conjugated polyclonal antibody (Dako), and then washed and developed with DAB/H₂O₂. The reactivity with human samples was detected by incubation of the blocked membrane with pooled culture-confirmed positive and negative serum samples for 1 h at 37 °C; the samples were washed, incubated with rabbit anti-human IgG HRP-conjugated polyclonal antibody (Dako), washed and then developed with DAB/H₂O₂. On appearance of a golden brown band at the site of reaction, the membranes were washed twice with distilled water to stop the enzymic reaction and scanned.

Agglutination assays for antibody detection. All 158 serum samples were subjected to RBPT. Briefly, 50 µl serum was pipetted onto a clean microscope slide. A drop of RBPT antigen was added and mixed with a sterile tooth pick and observed for an agglutination reaction. STAT was also carried out with 2-mercaptoethanol following a standard protocol (Alton *et al.*, 1975).

Indirect microplate ELISA for IgG antibody detection. An indirect microplate IgG ELISA was standardized using the purified rOmp28. The protein was diluted from the stock solution to a concentration of 250 ng µl⁻¹ in 0.1 M carbonate buffer (pH 9.6) and further coated on to Nunc immunoplates/modules. Briefly, each well was coated with 100 µl diluted protein and incubated at 37 °C for

2 h, washed with PBS and then blocked with 200 µl 1 % BSA in PBS at 4 °C overnight. The blocked wells were washed twice with PBS and stored at 4 °C until further use. The patient samples were diluted 1:1000, 100 µl was added per well and the plates were incubated at 37 °C for 1 h. After incubation, the wells were washed three times with 300 µl PBS-T and incubated with HRP-conjugated anti-human IgG (1:1000 dilution), and then washed again three times with 300 µl PBS-T. The wells were developed with 100 µl developing solution consisting of *o*-phenylenediamine and H₂O₂, and kept in the dark for 5 min for colour development. The peroxidase reaction was stopped by the addition of 25 µl 0.5 M H₂SO₄, and the absorbance was read at 490 nm in an ELISA reader. To ensure uniformity in testing and that repeated performance tests were consistent, one culture-confirmed positive sample and one culture-confirmed negative sample were run in duplicate on each plate. Each sample was run in duplicate and the mean absorbance value was used for further analysis.

Dot-blot ELISA for antibody detection. The rOmp28 protein was suspended in 200 µl 0.1 M carbonate buffer (pH 9.6) at a concentration of 5 µg µl⁻¹, and 2 µl of this antigen was coated in the middle of a nitrocellulose membrane fixed on plastic combs and allowed to dry at 37 °C for 1 h. Non-specific sites were blocked with 1 % BSA in PBS overnight at 4 °C. The combs were washed three times with PBS and incubated with different samples diluted 1:100 in PBS for 1 h at 37 °C. The combs were washed three times with PBS and incubated for 1 h at 37 °C with HRP-conjugated anti-human IgG diluted 1:500 in PBS. The strips were then again washed three times with PBS and the reaction was developed with DAB/H₂O₂ substrate solution. Rinsing the combs in distilled water stopped the reaction, and the results were read visually after complete drying of the nitrocellulose membrane. In the 12-legged combs, the last two spots were incubated with a culture-confirmed negative and positive sample to ensure uniformity in test results; the remaining ten spots were used for samples. The correlation, sensitivity, specificity and positive and negative predictive values were calculated using the formulas described in Table 1. In the calculations, RBPT and STAT results were taken as the standard test, and the dot and plate ELISA results were compared with those of these agglutination assays. A true positive was defined as a sample positive by the respective agglutination tests, as well as by the rOmp28 ELISA, and a true negative sample was negative by both tests evaluated. False-negative samples were classified as those that were positive by the agglutination test but negative by ELISA, and false-positive samples were those that were negative by the agglutination tests but positive by ELISA.

Table 1. Comparison of rOmp28 in the indirect plate ELISA and dot-blot ELISA with other agglutination tests routinely used in the laboratory for diagnosis of brucellosis

Calculation	rOmp28 indirect microtitre plate ELISA vs RBPT	rOmp28 dot-blot ELISA vs RBPT	rOmp28 indirect microtitre plate ELISA vs STAT	rOmp28 dot-blot ELISA vs STAT
Correlation (%) [*]	88.60	89.87	91.13	92.41
Sensitivity (%) [†]	97.50	82.05	97.72	95.69
Specificity (%) [‡]	85.59	92.43	88.50	88.09
Positive predictive value (%) [§]	69.64	78.04	76.78	88.09
Negative predictive value (%)	98.04	94.01	99.01	95.69

^{*}[(Number of samples positive by both methods + number of samples negative by both methods)/(total number of samples)] × 100.

[†][True positives/(true positives + false negatives)] × 100.

[‡][True negatives/(true negatives + false positives)] × 100.

[§][True positives/(true positives + false positives)] × 100.

^{||}[True negatives/(true negatives + false negatives)] × 100.

RESULTS AND DISCUSSION

Brucellosis is a zoonotic infection and is also recognized as an emerging public health disease that is endemic in most regions of the developing world. Diagnosis of this disease is carried out mostly by isolation and characterization of the bacterium from blood samples, but the bacterium is very slow growing and takes a minimum of 3–8 days, and sometimes as long as 45 days, to grow. The slow growth of *Brucella* in primary culture delays the diagnosis and treatment for at least several days, and the sensitivity of the culture technique is also very low and depends on many factors such as disease stage, culture medium and availability of viable bacteria in the blood sample and also the blood culture technique employed (Yagupsky, 1999; Mantur & Mangalgi, 2004). Hence, isolation of bacterium is not a feasible diagnostic tool for the confirmation of brucellosis. In the absence of an effective isolation procedure, serological tests, mainly agglutination tests, are relied on for the clinical diagnosis of brucellosis. Several studies have compared the tests available for the diagnosis of brucellosis and confirmed the superiority of ELISAs for detection in chronic and complicated cases (Araj *et al.*, 1986). In addition, when compared with the STAT, Coombs test and immunofluorescence assays, ELISAs were found to be simple, rapid and reliable (Araj *et al.*, 2005).

Expression, purification and reactivity testing of rOmp28

The cloning of the *omp28* gene in the pET-28a expression system led to the expression of a mature immunoreactive protein of approximately 32 kDa. On cloning in this system, an additional 174 bp from the vector corresponding to 58 aa including the 6 × His tag were also co-expressed with the complete Omp28 protein. The sequence of the cloned product was confirmed and found to match completely that of the reported sequence of the Omp28 gene for *B. melitensis* 16M (GenBank accession no. U30815.1). The other protein closely related to Omp28 is Omp25 of *B. abortus*, with these proteins having 43 % similarity (Lindler *et al.*, 1996). Expression was standardized with different concentrations of IPTG and different time durations after induction. The results suggested that an IPTG concentration of 1.5 mM and 5 h of incubation under shaking conditions after induction were optimum for expression of the protein. Purification was achieved under native conditions; recombinant protein that bound to the Ni-NTA agarose was eluted using a pH gradient. The different washes and eluates were analysed by SDS-PAGE and the protein was purified to greater than 90 % (Fig. 1a). The yield of the purified protein from the shake flask culture was calculated and was estimated at 14.15 mg protein l⁻¹. The immunoreactivity of the expressed protein was confirmed by Western blotting; the blots were reacted with anti-His antibody, rabbit polyclonal antibody raised against the whole-cell sonicated antigen of *B. melitensis* 16M and culture-confirmed human positive and negative

Fig. 1. Expression and purification of rOmp28 in a pET expression system. (a) SDS-PAGE analysis of protein expression in bacterial cell lysate. Lanes: 1, *E. coli* BL21(D3) cells induced for 5 h; 2, *E. coli* BL21(D3) cell lysate; 3, Ni-NTA agarose flow-through; 4, wash of Ni-NTA agarose; 5, pre-stained protein markers (MBI Fermentas); 6, wash of Ni-NTA agarose; 7, eluate 1; 8, purified recombinant protein. (b) Analysis of the reactivity of the expressed protein by Western blotting. Lanes: 1, pre-stained protein marker (MBI Fermentas); 2, anti-His antibody; 3, rabbit polyclonal antibody against whole-cell sonicated antigen; 4, culture-confirmed pooled positive human serum; 5, culture-confirmed negative human serum. (c) Dot-blot analysis. Samples: 1, 3, 4, 6, 9, human serum samples positive for brucellosis; 2, 5, 7, 8, 10, human serum samples negative for brucellosis; 11, culture-confirmed human negative sample; 12, culture-confirmed human positive sample.

serum samples. The protein band at ~32 kDa reacted with the rabbit polyclonal antibody, anti-His antibody and culture-confirmed human positive serum (Fig. 1b). No reaction was observed in the culture-confirmed human negative serum samples. The protein was found to be highly stable at 4, 15, 25, 37 and 45 °C, and at a pH of 4–9 in phosphate buffer for a period of 12, 24, 48 and 72 h,

with an intact single band observed by SDS-PAGE after incubation, and no observed degradation of the protein. Furthermore, when the protein was tested after incubation, it retained its immunoreactivity as confirmed by ELISA.

Immunoassay with clinical samples and comparative evaluation against standard agglutination tests

Overall, 158 serum samples collected from suspected cases of brucellosis and healthy individuals were subjected to the microtitre plate ELISA and dot-blot assay. The microtitre plate ELISA assay was considered positive only if the mean absorbance value was greater than three SDs above the mean value for the healthy controls. The mean absorbance value and SD for the healthy subjects studied was 0.15 ± 0.06 , and a sample was considered positive when the absorbance of the measurements in duplicate was greater than 0.33. Out of 158 samples tested, 56 (35.44 %) were positive and 102 (65.56 %) were negative by microtitre plate ELISA, and, in comparison with the RBPT, the sensitivity and specificity were 97.50 and 85.59 %, respectively, whereas when compared with STAT the sensitivity and specificity were 97.72 and 88.50 %, respectively (Table 1). Thus, the test gave comparable results with the two standard agglutination assays. It is important to note that 17 samples that were negative by RBPT and 13 samples negative by STAT were detected as positive by the microtitre plate ELISA, suggesting that low-titre samples that are missed by the agglutination tests were detected by the sensitive ELISA. In the case of the dot-blot assay, appearance of a visible dot was considered positive, whilst no visible dot was considered negative. When the test was completed on all 158 samples, 41 samples (25.95 %) were positive and 117 (74.05 %) were negative for brucellosis (Table 1). Similarly, when compared with the agglutination assays, in the dot-blot assay, nine samples that were negative by RBPT and five samples negative by STAT were detected as positive in the dot-blot assay. The sensitivity and specificity of the dot-blot assay in comparison with RBPT was 82.05 and 92.43 %, and in comparison with STAT was 95.69 and 88.09 %, respectively.

All serum samples were grouped as group I, II or III, and the RBPT and STAT results of the grouped samples are given in Table 2. The absorbance values of each of the samples in all three groups were analysed based on the RBPT and STAT results, and are shown in Fig. 2. In group I, for which bacteriological results were also available, all the culture-positive samples gave absorbance values greater than 0.33 and all the culture-negative samples had absorbance values that were much lower than 0.33. In group II, comprising clinically suspected cases, the RBPT- and STAT-positive samples also had much higher absorbance values in comparison with the negative samples. In the group III serum samples, collected from healthy volunteers, 79.4 % of the samples had absorbance values

Table 2. Results of bacterial isolation and RBPT and STAT in different groups

Test result	Group I	Group II	Group III
No. of samples positive for bacterial isolation*	6	ND	ND
No. of samples negative for bacterial isolation	27	ND	ND
RBPT positive	6	34	0
RBPT negative	27	57	34
STAT positive	6	38	0
STAT negative	27	53	34
Total (n)	33	91	34

ND, Not determined.

*All cultures were positive for *B. melitensis* and were confirmed by biochemical tests.

of less than 0.1 in both the RBPT- and STAT-negative samples. None of the samples from the healthy volunteer group was found to be positive by either RBPT or STAT.

Several proteins have been identified as potential markers for disease diagnosis of brucellosis, as antibodies against these markers have been demonstrated in culture-confirmed positive human serum samples (Eschenbrenner *et al.*, 2002; Wagner *et al.*, 2002; Connolly *et al.*, 2006). In addition, antibodies against a few recombinant antigens, such as Omp31, have been studied and identified as potential diagnostic markers (Vizcaino *et al.*, 1996). Several antigens have been used in serological tests for brucellosis; most of the antigens were in the form of whole cells, cell sonicated extracts or LPS or proteins extracted from whole-cell sonicated extracts. All these forms share common antigens among the different *Brucella* species, and serological tests directed against these antigens can detect all species except *Brucella canis* and *Brucella ovis* (Al Dahouk *et al.*, 2003). *Brucella* group 3 antigens are OMPs with a molecular mass of 25–30 kDa and are known to be similar to *E. coli* OmpA protein (Marquis & Ficht, 1993). The major OMPs react with all *Brucella* species including *B. canis* and *B. ovis* (Cloeckaert *et al.*, 2002). Among the different antigens, major OMP proteins of 25–27 and 36–38 kDa, and minor OMP proteins of 17 and 10 kDa, have been identified (Zygmunt *et al.*, 1992). The cell envelope proteome of *B. abortus* has been analysed and found to consist of 164 proteins, and based on reactivity with positive sera from cows and humans, reactive proteins including Omp31, Omp2b porin and GroEL have been identified (Connolly *et al.*, 2006). The preparation of these antigens from *Brucella* species requires precautions against biosafety hazards. The commercial kits available for the diagnosis of brucellosis are not popular in the routine

Fig. 2. Comparison of Omp28 ELISA with RBPT (i) and STAT (ii) results, showing the distribution of absorbance values in different groups. (a) Group I, bacteriologically confirmed ($n=33$); (b) group II, clinically suspected ($n=91$); (c) group III, healthy volunteers; (d) all samples tested ($n=158$). Black bars, negative results; grey bars, positive results.

diagnosis of brucellosis in the developing world due to their high cost and the lack of easy availability.

A survey of the literature has shown that rOmp28 produces a humoral immune response in culture-confirmed human sera for both *B. abortus* and *B. melitensis*, and has been suggested as a vaccine candidate for brucellosis (Lindler *et al.*, 1996). In the present study, we demonstrated that Omp28 is a candidate for the serodiagnosis of brucellosis and should be

explored further as a potential candidate for the development of an antigen detection system for human brucellosis. Any diagnostic kit developed with this protein needs to be able to survive harsh environmental conditions in tropical climates where it will be used the most. In order to achieve this, the stability of the protein was evaluated following storage under different temperature and pH conditions. The protein was found to be stable at pH 4.0–9.0 and at temperatures from 4 to 45 °C; after storage, the protein remained intact and its

reactivity with antibodies was preserved, confirming that this recombinant protein is a suitable candidate for the development of a diagnostic system.

The recombinant antigen was also tested on a number of human samples that were positive for malaria and typhoid. The absorbance values of all 14 samples positive for malaria was less than 0.12, and 12 samples positive for typhoid were less than 0.1. In the case of the dot-blot ELISA, no dot was observed in either the malaria- or typhoid-positive samples. This suggests that the test is highly specific and that no cross-reactivity exists with agents of other fever-related illness.

Evaluation of the indirect microtitre plate ELISA against the agglutination tests showed a correlation of 88.60 % with RBPT and 91.13 % with STAT. The high sensitivity and specificity of this rOmp28 ELISA in comparison with RBPT and STAT reiterates the fact that this system can be used for the routine serodiagnosis of brucellosis in the field. As this system is in a microtitre plate ELISA format, it allows batch processing so that large numbers of samples can be screened at the same time. In addition, it is less time-consuming and the results can be analysed quantitatively using an ELISA reader. The dot-blot assay also had a high correlation, sensitivity and specificity in comparison with RBPT and STAT. The dot-blot format is highly suitable for field use to provide a cost-effective test with prompt results where laboratory facilities and equipment such as ELISA readers are not available. In the absence of a reliable antigen detection system and because of the problems associated with performing PCR-based diagnostic tests in peripheral and secondary-level hospitals in endemic areas, serology remains the mainstay in the diagnosis of brucellosis. The development of ELISA-based diagnostic techniques that use highly specific recombinant proteins will not only allow cost-effective diagnosis but will also provide reliable field-based methods, in addition circumventing the necessity to handle pathogenic *Brucella* species for antigen preparation.

In conclusion, rOmp28 was found to be highly reactive with most of the positive serum samples, confirming the immunogenic nature of this protein, and may help in the development of an antigen detection system. This antibody detection system requires further evaluation on a large number of clinical samples from different geographical areas and with various clinical presentations. The system should also be compared with some of the commercially available ELISA systems such as the Panbio ELISA kit and the Brucellacapt test (Viracell). In conclusion, a diagnostic test developed with this recombinant antigen should not only provide high sensitivity and specificity but also take into account the safety aspects associated with handling *Brucella* species in the laboratory.

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