ELISA FOR BRUCELLOSIS DETECTION BASED ON THREE *BRUCELLA* RECOMBINANT PROTEINS

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Abstract. Control of brucellosis among farm animals, wildlife and humans require reliable diagnosis. Rose Bengal serological test (RBT) is based on lipopolysaccharide antigen of *Brucella*, which may cross react with other gram-negative bacteria and produce false positive result. Immunoreactive proteins, such as outer-membrane protein BP26, ribosome recycling factor protein CP24 and *Brucella* lumazine synthase (BLS), previously reported to be recognized by infected sheep sera, were selected for production of recombinant proteins for use in an ELISA in order to investigate immune response among goats and cows, in comparison with commercial RBT. Cut-off value for ELISA was based on the immune response of *in vitro* fertilized goats and cows. Goats positive for *Brucella* culture or by RBT were ELISA positive for either IgG or IgM against at least one recombinant protein. For animals with negative RBT, animals with positive ELISA could be detected, and 61.6% possessed ELISA values as high as in infected animals. Thus, this ELISA procedure is proposed as an alternative to RBT for screening of brucellosis in farm animals.

Keywords: brucellosis, diagnosis, ELISA, recombinant protein

INTRODUCTION

Brucellosis is a major zoonotic disease of public health and animal welfare, and is of economic significance worldwide (Georgios and Nicolaos, 2005). The disease is caused by members of the genus *Brucella* (Cutler and Cutler, 2006; Rajashekara *et al*, 2004), and brucellosis presents a great variety of clinical manifestations, which makes it difficult to diagnose based on clinical signs. Therefore, diagnosis must be confirmed directly by isolation of *Brucella*, mostly from blood culture or indirectly by detection of immune response against its antigens (Orduña *et al*, 2000).

At present, diagnosis of brucellosis is performed using serological techniques mainly based on the detection of antibodies against lipopolysaccharide (LPS) of smooth strain *Brucella* (Weynants *et al*, 1997). However, one of the major drawbacks in employing *Brucella* LPS is the substantial similarity of its O-polysaccharide to that of various other gram-negative bacteria, such as *Escherichia*

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coli O:116 and O:157, *Salmonella* group N (O:30) of Kaufmann-White, *Pseudomonas multophilia, Vibrio cholerae* and especially *Yersinia enterocolitica* O:9 (Cutler *et al*, 2005; Muñoz *et al*, 2005). Consequently, this results in serological cross reactivity and leads to low specificity.

Antigen preparation is from whole cell preparation or crude cell extract derived from *Brucella* and Rose Bengal test (RBT) is routinely used. These antigen preparations are hazardous, as they can cause disease directly through contaminated aerosols (Samartino *et al*, 1999). In eradication programs, veterinarians cannot differentiate vaccinated from infected animals using LPS-based assays (Baldi *et al*, 2000). In addition, *Brucella* remains on the Center for Disease Control and Prevention (CDC) category B list of potential biological warfare agents (Pappas *et al*, 2006).

Therefore, an important development goal in diagnosis of brucellosis is the identification of protein antigens that induce a robust antibody response during infection. Antigens shared by all *Brucella* species would be of extreme importance for diagnostic purposes. A few such antigens have been described in recent years. For example, *Brucella* lumazine synthase (BLS), an 18-kDa cytoplasmic protein present in all *Brucella* species, has been used in the diagnosis of human and canine brucellosis (Goldbaum *et al*, 1993, 1999). Another antigen found in all *Brucella* species is a 24-kDa protein known as CP24, a homologue of *E. coli* ribosome recycling factor (RRF) and other species, and is present in the cytosol of both smooth and rough *Brucellae* (Cassataro *et al*, 2002a,b). CP24 protein is recognized by sera from sheep experimentally infected with *B. melitensis* but not by sera from animals vaccinated with *B. melitensis* attenuated strain Rev-1 (Cassataro *et al*, 2002a,b). In addition, recombinant CP24 protein heterologously expressed in *E. coli* is recognized by sera from *Brucella*-infected sheep (Vizcaíno *et al*, 1996). Another non-LPS group of antigens employed for diagnostic purposes is that of the outer membrane proteins (OMPs) of *Brucella* spp. Of most interest is BP26 or Omp28 protein, which has been identified simultaneously by three research groups as an immunodominant antigen in infected cattle, sheep, goat, and humans (Cloeckaert *et al*, 1996; Lindler *et al*, 1996; Rossetti *et al*, 1996). BP26 is named according to the nomenclature of Rossetti *et al* (1996) who was the first to publish the nucleotide sequence of *bp26*. BP2 is capable of detecting brucellosis in sheep caused by *B. melitensis* or *B. ovis* by means of competitive enzyme-linked immunosorbent assay (ELISA) using BP26-specific monoclonal antibodies (MAbs) (Lindler *et al*, 1996) or by an indirect ELISA using the protein partially purified from *Brucella* spp (Letesson *et al*, 1997), which provides good results in differentiating between *B. melitensis*-infected and *B. melitensis* Rev.1-vaccinated sheep.

This study describes the production of three recombinant proteins, namely, rBP26, rCP24 and rBLS. Immunogenicity of the recombinant proteins was studied by mouse immunization, and subsequently, immunized mouse sera were subjected to reaction with native *Brucella* proteins in order to demonstrate that the epitopes of the three recombinant protein*s* were shared with those of *Brucella* endogenous proteins. These recombinant proteins were also evaluated for their immunological reactivity to sera from normal and infected animals using western blot analysis and ELISA.

MATERIALS AND METHODS

Bacterial strains and reagents

Brucella strain used in the study was a Thai *B. melitensis* isolate, identified and confirmed by multiplex PCR (Benjathummarak and Kalambaheti, 2012). *B. melitensis* was isolated on *Brucella* agar (tryptic soy agar containing *Brucella* supplement (Oxoid, Hamshire, UK) and 6 antibiotics. Expression vectors, pRSET A, B and C, and *E. coli* BL21(DE3) containing pLysS and competent *E. coli* DH-5α strains employed as host cells for the recombinant plasmid-constructions were from Invitrogen (Paisley, UK).

Sera samples

Goat and bovine sera were randomly collected from farms and subjected to RBT in order to determine the presence of antibodies to *Brucella* cell wall. Vaginal swabs were collected from both seropositive and -negative animals, based on both *Brucella* culture results and RBT. Five groups of animal sera were included for ELISA tests: 7 goat sera with positive cultures, 8 goat sera positive with RBT, 60 goat sera with negative RBT, 6 cow sera with positive RBT, and 54 cow sera with negative RBT. All animal protocols were approved by the Faculty of Tropical Medicine, Mahidol University Animal Care and Use Committee.

DNA cloning

Recombinant pRset expression plasmids, each containing a target gene were constructed as follows. The primers for each target gene were designed so as to contain a specific restriction enzyme site was presented in each primer (Table 1). PCR (25 µl) contained 50-100 ng of *Brucella* genomic DNA, 1X PCR buffer, 200 µM each dNTP, 1 U *Taq* DNA polymerase, and 10 pmol of each primer pair. PCR

Table 1
Primers used in construction of recombinant *Brucella* proteins.

Protein (locus taq of *Brucella melitensis* bv 1 str 16M genome)	MW; kDa	Primer (5'_3')	DNA strand	Corporated restriction enzyme size	Size of PCR product (bp)
Lumazine synthase"ribH" or BLS; BMEII0589	17.22	TATGAACTCGAGATGTCCGAACAAGACA	+	*Xho*I	483
		TTAGTCGGAATTCCGCGGCGATGCGGCTG	-	*Eco*RI	
Bp26; BMEI0536	26.42	ACGCAGCCTCGAGGCATCGCCGTCACCGG	+	*Xho*I	753
		ATTGACCCATGGTTATAGCTGTTTTCG	-	*Nco*I	
Cp24;uridylate kinase or ribosome recycling factor BMEI0825	20.72	TTTGACTCGAGAGACCTGAAACGCCGCA	+	*Xho*I	840
		CTGCATGAATTCCCCCTCCTTGACAGCA	-	*Eco*RI	

amplicon and cognate plasmid vector were digested with the same restriction enzyme and subjected to ligation overnight. *RibH2* and *CP24* fragments were inserted into pRSET-B, while *BP26* fragment was inserted into pRSET-C. Each recombinant plasmid was then transfected into competent *E. coli* DH5α and transformants selected under ampicillin (100 µg/ml) and confirmed by PCR. Recombinant plasmid was then used to transform *E. coli* BL-21(DE3) containing pLysS under chloramphenicol (40 µg/ml) selection.

Heterologous expression and purification of recombinant proteins

Exponential-phase cultures of transformants containing recombinant plamids were treated with 1 mM IPTG for 3 hours. Induced cells, as well as un-induced cells, were lyzed by ready-mix lysis buffer containing 0.1% triton X-100 and 20 µg/ml lysozyme; Bugbuster (Novagen, Madison, WI) and centrifuged at 10,000*g* for 20 minutes at 4ºC. The pellets were subjected to sonication for 5 minutes with a pulse interval of 5 seconds by sonicator (Sonics, Newtown, CT) and then centrifuged at 10,000*g* for 20 minutes at 4ºC. The supernatants and bacterial cell lysates were analyzed by western blotting. In brief, proteins from SDS-PAGE were electrophoretically transferred onto PVDF membranes (Bio-rad, Hercules, CA), which then were incubated with 3% bovine serum albumin in TBS buffer (0.1M Tris buffer saline pH7.4) overnight at 4ºC, washed with TBS containing 0.05% Tween 20, incubated with anti-His alkaline phosphatase (AP)-conjugated antibodies (Southern Biotech, Birmingham, AL), washed and His-tagged bands visualized using nitro blue-tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate substrate. Western blotting was also conducted using goat sera and mouse anti-goat IgG AP-conjugated polyclonal antibodies (Southern Biotech, Birmingham, AL).

After confirmation of solubility, recombinant proteins were purified by Ni-NTA agarose affinity column (QIAGEN, Hilden, Germany). The His-tagged fusion proteins were eluted by a gradient of imidazole ranging from 0 to 0.5 M. The purified proteins were analyzed by SDS-PAGE and protein concentration was measured using Bio-rad Bradford protein assay. The purified proteins were dialyzed against 0.01 M phosphate-buffered saline (PBS) pH 7.2 and lyophilized.

Mouse immunization

Five male ICR mice from National Laboratory Animal Center, Mahidol University were injected intraperitoneally twice, at a two-week interval, with 10 µg of recombinant protein mixed at 1:1 ratio with Imject® Alum (Pierce Biotechnology, Rockford, IL). Five days after the last injection, mice were bled via the eye and serum antibody-titer against each recombinant protein antigen was determined by ELISA. Blood was collected from mice with elevated antibody titers and sera were reacted by western blotting with *Brucella* whole cell lysate.

RBT

A 25 µl aliquot of serum was pipetted onto a clean microscope slide and an equal volume of RBT antigen (BENGATEST; Synbiotics, San Diego, CA) was added. After mixing the agglutination reaction was recorded.

Indirect ELISA detection of IgG and IgM

In brief, to each well of a micro-titer plate (Nunc, Roskilde, Sweden) was added 100 µl aliquot of varying concentrations of recombinant proteins diluted in 0.1 M

carbonate buffer (pH 9.6) and the plates were incubated at 4°C overnight. Each plate was then washed with 0.1M PBS pH7.2 containing 0.05% Tween-20 (PBS-Tween) and incubated with 200 µl aliquot/well of 1% bovine serum albumin (BSA) in PBS-Tween at 37°C for 1 hour. Wells were washed with PBS-Tween, 100 µl aliquot of serum samples diluted in PBS containing 0.2% gelatin and 0.2% BSA was added to each well and plates were incubated at 37°C for 1 hour. Wells were washed with 200 µl aliquot of PBS-Tween and incubated with AP-conjugated anti-goat IgG or AP-conjugated anti-goat IgM (1:4,000 dilution), while AP-conjugated anti-cow IgG (1:4,000 dilution) was employed for cow sera. Plates were incubated for 1 hour, washed as described above and each well was treated in the dark for 1 hour with 100 µl aliquot of developing solution consisting of *p*NPP substrate (KPL, Gaithersberg, MD). The phosphatase reaction was terminated by addition of 50 µl aliquot of 5% EDTA solution and optical density (OD) at 405 nm was measured in an ELISA reader (Tecan Group, Männedorf, Switzerland). In order to ensure accuracy of ELISA assay, control sera (one goat serum positive for *Brucella* culture and one negative for culture) were included in each plate. Each serum was tested in duplicate and data recorded as mean OD.

RESULTS

Cloning of *Brucella* genes

PCR amplicons of each target gene contained two different restriction enzyme sites at each primer pair: *Xho*I and *EcoR*I for *rib*H2 and *Cp*24, and *Xho*I and NcoI for *Bp*26. Amplicons were digested with their respective enzymes, ligated to plasmid vectors similarly treated, pRsetB for *rib*H2 and *Cp*24, and pRsetC for *Bp*26, and cloned in *E. coli* DH5a and subsequently in *E. coli* BL-21(DE3) containing pLysS for heterologous expression. The recombinant plasmids contained 483, 630 and 545 bp inserts of *rib*H2, *Bp*26 and *Cp*24, respectively (data not shown).

Expression and purification of *Brucella* recombinant proteins

Following induction of protein expression with 1 mM IPTG for 3 hours, bacterial cell lysates and supernatants from sonicated cell pellets were analyzed by western blotting using anti-His tag antibodies, revealing rBP26, rBLS and rCP24 fragments of 35, 23 and 24 kDa, respectively (data not shown). As the majority of the heterologously expressed recombinant proteins were in the soluble fraction (bacterial cell lysate), they were then purified using Ni-NTA affinity chromatography. RBP26 was eluted with 50 mM imidazole (Fig 1A), and rBLS (Fig 1B) and rCP24 (Fig 1C) with 2 mM imidazole.

Reactivity of mice anti-recombinant proteins immune sera against *Brucella* whole cell lysate

The immunization regimen produced mice immune sera with reactivity to each recombinant protein of titers > 1:128,000 as determined by ELISA. Immune sera to the three purified recombinant proteins were then tested against *Brucella* whole cell lysate in order to evaluate their specificities against the natural antigens, which should be 3-5 kDa smaller than their corresponding recombinant antigens. At 1:20,000 dilution, mouse anti-rBP26 (35 kDa) immune serum recognized a 26 kDa protein from *Brucella* whole cell lysate (Fig 2A), that raised against rBLS (23 kDa) recognized a 13 kDa antigen (Fig 2B) and anti-rCP24 (24 kDa) immune serum

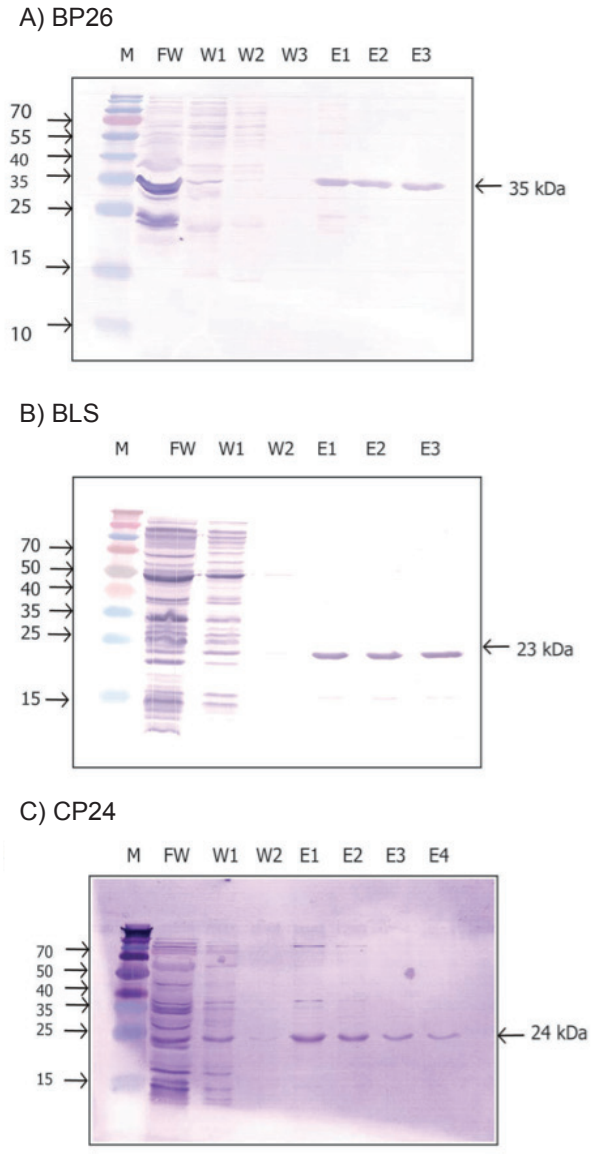

Fig 1–Purification of histidine-tagged fusion recombinant proteins. Bacterial cell lysates containing *Brucella* rBP26, rBLS and rCP24 were purified using a Nickle-NTA affinity chromatography as described in Materials and Methods. 1A, rBP26. 1B, rBLS. 1C, rCP24. FW, first wash solution; W, washed fraction; E, eluted fraction using the appropriate imidazole solution.

reacted with a 20 kDa antigen (Fig 2C).

Immunoreactivity of recombinant *Brucella* proteins to goat sera using western blotting

Western blot assays were performed to test the immunoreactivities of the three recombinant *Brucella* proteins against two groups of goat sera, RBT-positive and RBT-negative. Each purified recombinant protein was individually blotted onto PVDF membrane at 200 µg per large strip, which was then cut into smaller strips for testing with individual goat serum diluted 1:100. All three recombinant proteins were able to react with the 8 RBT-positive and 30 RBT-negative sera (data not shown).

Immunoreactivity of recombinant *Brucella* proteins to goat sera using ELISA

Optimized concentration of the three recombinant proteins determined against a goat serum positive by RBT and *Brucella* culture was 125 ng/ml for BP26 and BLS, and 250 ng/ml for CP24. A serum dilution of 1:1,000 and 1:200 was selected for IgG and IgM detection, respectively. The test group consisted of 7 goats positive by *Brucella* culture, of which 6 were RBT-positive, 2 goats positive by RBT but negative by *Brucella* culture and 61 goats with negative RBT.

In order to establish the ELISA procedure, the cut-off level of antibody response was based on the OD value obtained from sera of goats conceived by *in vitro* fertilization, screened for absence of maternal anti-*Brucella* antibodies and raised in a closed farm. For determination of IgM cut-off level, the mean OD + SD at 405 nm of 10 negative goat sera to rBP26, rBLS and rCP24 of 0.337, 0.318, 0.416, respectively, were used (Fig 3). Of the 6 infected goats, positive by *Brucella* culture and RBT, their IgM levels were as low as in the negative goats. Among goats negative by RBT, 25 (41.67%) showed OD values higher than

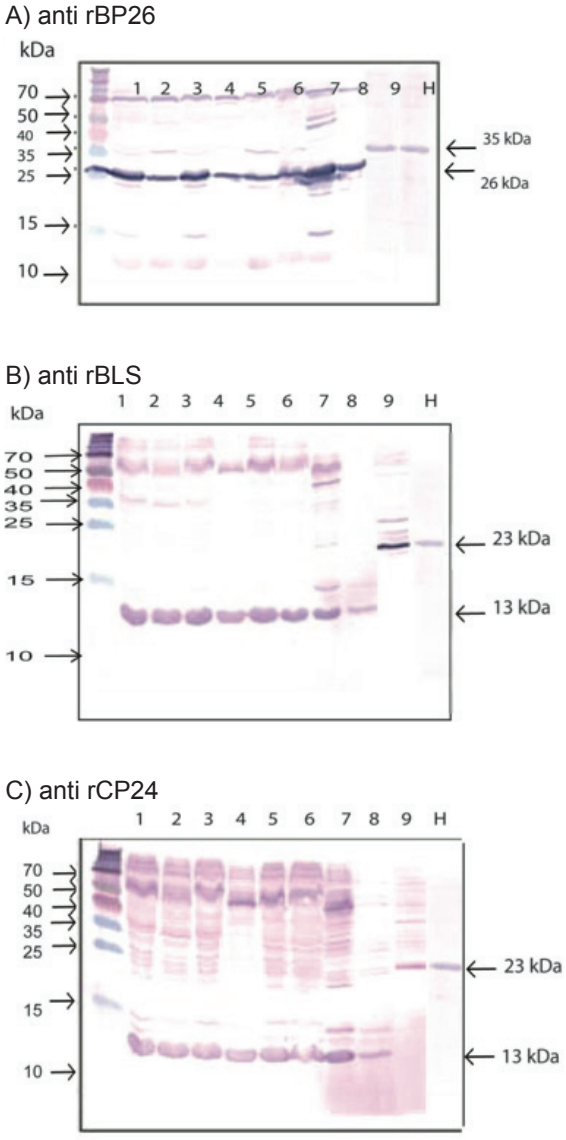

Fig 2–Immunoreactivity of mice antisera raised against purified recombinant proteins to *Brucella melitensis* whole cell lysate by western blotting. 2A, reactivity of mouse anti-rBP26 immune serum. 2B, reactivity of mouse anti-BLS immune serum. 2C, reactivity of mouse anti-rCP24 immune serum. Lanes 1-8 were isolated strains of *B. melitensis* in Thailand; lane 9, purified recombinant protein treated with its own mouse immune serum; lane H, purified recombinant protein treated with mouse MAb to His-tag.

the cut-off level. These trends were the same for all three recombinant proteins.

For IgG determination, the cut-off level for rBP26, rBLS and rCP24 was 0.545, 0.585 and 0.619, respectively (Fig 4). Among 9 infected goats, of which 7 were positive for *Brucella* culture, only 2 were ELISA positive (Table 2). In this study ELISA positivity was judged from the cut-off OD values of the recombinant antigens, and positive immunoreactivity to at least one recombinant protein was assigned as being ELISA positive. When results from both IgM and IgG levels against all three recombinant proteins were included for evaluation, only one goat serum showed negative ELISA result, although it was positive by culture and RBT, while the rest in the group were ELISA positive.

The same ELISA-cut off criterion was then applied to the 60 RBT-negative goats: 25 had positive IgM and 37 positive IgG values for at least one recombinant protein. In general, goats that were positive for IgM were IgG negative, except for two sera, which were both IgG and IgM positive for at least one recombinant protein.

Immunoreactivity of recombinant *Brucella* proteins to cow sera

Only IgG was determined for cow sera, of which 6 were positive and 53 negative in RBT. However, among the latter group, IgG levels to the recombinant proteins were low and the mean OD + 2 SD was employed as the cut-off value. Thus in cows, the cut-off OD value of IgG to rBP26, rBLS and rCP24 was 0.230, 0.261 and 0.215, respectively.

The 10 cows conceived by *in vitro* fertilization had IgG response to the three recombinant proteins, as measured by ELISA, of approximately 0.1 OD, and so the cut-off value was thus assigned as

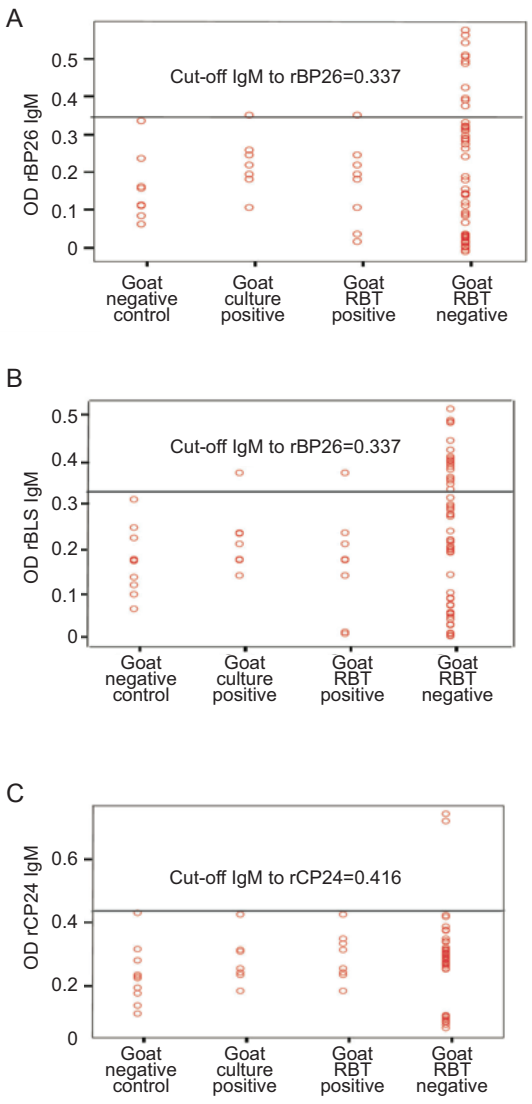

Fig 3–IgM response to recombinant proteins using ELISA. ELISA OD values were obtained from goat sera at 1:200 dilution. The test groups consisted of negative control goats conceived by *in vitro* fertilization, screened for absence of maternal antibodies and reared in a closed farm; goat positive for *Brucella* culture; goat positive for RBT; and goats negative for RBT. Cut-off OD value is marked for each recombinant protein, based on mean + 2 SD OD value of negative control group.

mean + 2 SD, with cut-off value for anti-rBP26, -rBLS and -rCP24 IgG of 0.230, 0.261 and 0.215, respectively. Of the 60 cow sera collected from farms where the animals were allowed to graze freely, only 6 samples were RBT positive but all were IgG-positive to all three recombinant proteins as expected. The 54 cows negative for RBT were highly IgG-positive to all three recombinant proteins, except for one single serum.

DISCUSSION

Identification of protein antigens that are useful for diagnosis and possibly for distinguishing the immunological response of infected animals from that of animals vaccinated with live attenuated *Brucella* strains, to which antibodies are targeted to LPS are needed. Identification of such protein constituents of *Brucella* that elicit an antibody response during infection would improve diagnosis of brucellosis and contribute to the development of new vaccine strategies.

Among the immunodominant antigens, Blast_P-homology search revealed that BP26, an outer membrane protein, and cytoplasmic BLS and CP24 proteins contained amino acid sequences conserved among *Brucella* spp. Mouse immune sera raised against heterologously expressed and affinity purified His-tagged rBP26 (35 kDa), rBLS (23 kDa) and rCP24 (24 kDa) recognized native BP26 and BLS of smaller than the expected sizes, except for CP24 (20 kDa). Native BP26 and BLS may have been degraded during the whole cell lysate preparation. However as all mouse antisera reacted with epitopes of the native proteins, albeit of smaller than expected size, these *Brucella* recombinant proteins were employed as antigens for western blot and ELISA-based detections

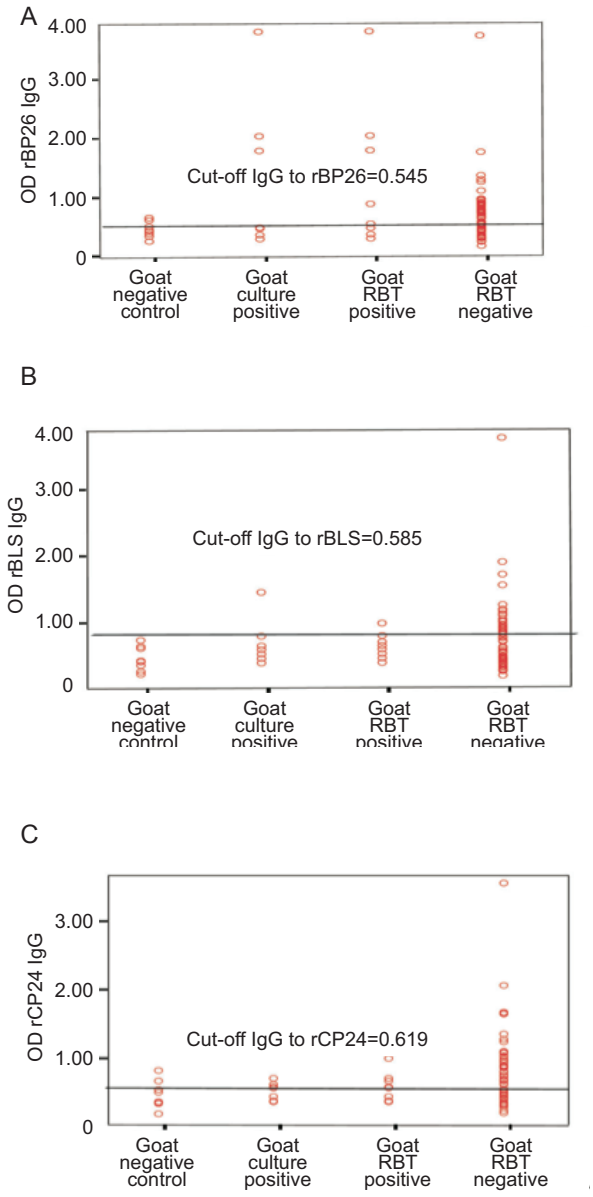

Fig 4–IgG response to recombinant proteins using ELISA. ELISA OD values were obtained from goat sera at 1:1,000 dilution. The test groups were as described in legend to Fig 3. Cut-off OD value is marked for each recombinant protein, based on mean + 2 SD OD value of negative control group.

of goat and cow sera. However, as sera from both RBT-positive and -negative goat and cow sera recognized all three recombinant proteins by western blotting, therefore subsequent efforts were directed in optimizing ELISA procedure.

In developing any diagnostic test, control negative animals are essential for comparative analysis. *In vitro* fertilized animals were chosen, which had no maternal antibodies against *Brucella* and were reared in a closed and clean house with no exposure to the environment. Using sera from such animals, optimized conditions for coating Ag, serum dilution and ELISA cut-off values for both IgM and IgG against the three *Brucella* recombinant proteins were established. A single serum dilution (1:200) was employed.

Among *Brucella*-infected goats, 70% had positive IgG response to at least one recombinant protein, 10% were positive for IgM to rCP24, and one goat (11%) was negative by ELISA. Thus both IgG and IgM responses could be used to detect brucellosis infection. Based on IgG response to recombinant proteins, 4, 5 and 3 out of 9 goats were positive to rBP26; rBLS and rCP24, respectively and thus IgG response to all three recombinant proteins should be included for a more accurate ELISA procedure. Cassataro *et al* (2002a) reported an increased detection in subacute and chronic brucellosis cases using BLS and CP24 together in an ELISA technique.

As goats and cows are reservoir hosts of *Brucella* spp, infected animals may not present any clinical signs as in human brucellosis, and infected animals may appear normal until abortion occurs (Godfroid *et al*, 2010). IgM levels to the three recombinant proteins were investigated in goats negative by RBT. Out of 60 goats, 41.6%

Table 2
Evaluation of ELISA-based procedure using recombinant proteins for detection of *Brucella*-infected goat.

Sample No.	BP26		BLS		CP24		
Cut-off OD	IgM 1:200 0.337	IgG 1:1,000 0.545	IgM 1:200 0.318	IgG 1:1,000 1:1,000 0.585	IgM 1:200 0.416	IgG 1:1,000 1:1,000 0.619	ELISA-validation
1CR	0.350	0.331	0.370	0.567	0.423	0.549	Positive
2C	0.258	0.465	0.233	1.421	0.307	0.603	Positive
3CR	0.244	0.268	0.235	0.361	0.311	0.366	Negative
4CR	0.106	1.767	0.138	0.768	0.182	0.687	Positive
5CR	0.217	2.007	0.209	0.425	0.240	0.350	Positive
6CR	0.193	0.441	0.172	0.614	0.253	0.567	Positive
7CR	0.181	3.790	0.175	0.496	0.233	0.422	Positive
8R	0.006	0.935	0.034	0.960	0.348	0.982	Positive
9R	0.010	0.508	0.016	0.677	0.332	0.648	Positive

C, Culture positive; R, Rose Bengal positive.

were IgM positive, and 2 animals with the highest IgM levels were positive to cytoplasmic proteins (rBLS and rCP24) and not the outer membrane protein (rBP26), suggesting that IgM was elicited during infection and cytoplasmic proteins are appropriate detecting-antigens. The number of positive cases for IgM response to rBLS was higher than rCP24, suggesting that IgM level might be detected by employing rBLS only, when those sera were positive to both rCP24 and rBLS. However there were a few cases of sera, that were positive to rCP24 only.

RBT could detect only 88% of positives *Brucella* cases, and 70% of RBT-negative goat sera had immune response of either IgG or IgM to at least one of the three recombinant proteins, except for 2 sera (3%) that were positive for both IgM and IgG. Similarly, among cows raised in open farms, all had positive immunoreactivity to each recombinant protein, except for only one cow, kept in a closed farm that had immunoreactivity similar to control negative animals. Thus our ELISA method was able to identify goats and cows in endemic area, which are reservoirs of *Brucella*, owing to their exposure to soil-borne *Brucella*. Abortion in these animals could occur whenever *Brucella* successfully evades the host immune response.

Chaudhuri *et al* (2010) has employed BP26/omp28 in ELISA to determine antibody response among cattle, goats and dogs. Delpino *et al* (2003) could not apply CP24 in ELISA test as the antibody response of the their *Brucella*-free animal was as high as in brucellosis animal.

RBT is the routine serological diagnostic method for brucellosis. As the major antigen of this agglutination test is LPS, vaccinated animals would already have antibodies positive to RBT. Thus detection of antibody response to proteins

recognized by the immune response during *Brucella* infection is more preferable. The ELISA procedure reported in this study employed three *Brucella* recombinant proteins, BP26, BLS and CP26, as antigens to detect IGM and IgG in goat sera and IgG in cow sera. The critical negative control sera were obtained from animals conceived by *in vitro* fertilization, screened for absence of maternal anti-*Brucella* antibodies and raided in a closed farm system. Criterion for an infected goat is ELISA-based positive antibody to at least one of the three recombinant proteins. Thus positive IgM response indicates that the animal has recently acquired *Brucella* antigen, while high IgG response indicates chronic *Brucella* infection. Our studies indicate that animals (goats and cows) in endemic areas can be identified as being reservoir hosts for *Brucella* if antibodies (IgM and/or IgG) to *Brucella* antigens are detected.

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