Specific Serodiagnosis of Animal Brucellosis Based on A Recombinant Multiepitope Protein Antigen

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Abstract

Background: Brucellosis is a zoonotic infectious disease that causes substantial public health problems and endangers the development of animal husbandry in endemic areas, causing huge losses of personal property. Early diagnosis of sick animals is a crucial step in reducing the incidence of brucellosis.

Objective: In this study, we designed a recombinant multiepitope protein (rMEP) as a serum diagnostic antigen for brucellosis and evaluated its diagnostic value in cattle and goats.

Methods: An indirect enzyme-linked immunosorbent assay (iELISA) was used to assess the new rMEP, and 159 goat and 153 bovine serum samples were measured, including brucellosis and nonbrucellosis samples. To better observe the effectiveness of rMEP, we performed receiver operating characteristic (ROC) curve analysis.

Results: Evaluation of the 159 goat serum samples showed that the area under the ROC curve (AUC) was 0.9976, and compared with serum tube agglutination test (SAT) and the Rose Bengal plate agglutination test (RBPT), the positive and negative diagnostic accuracies of ELISA were 98.92% (92/93) and 96.97% (64/66), respectively. Evaluation of the 153 bovine serum samples showed that the AUC was 0.9974, and compared with those of SAT and RBPT, the positive and negative diagnostic accuracies of ELISA were 98.65% (73/74) and 96.20% (76/79), respectively.

Conclusion: The results indicated that rMEP, as a protein antigen, can be used to diagnose brucellosis with high accuracy in both goats and bovines.

Introduction

Brucellosis is a chronic zoonotic disease of great public health significance caused by gram-negative, facultative, intracellular bacteria of the genus *Brucella*[1]. There are 6 classic species in the genus *Brucella*, namely, *B. abortus, B. melitensis, B. bovis, B. neotomae, B. suis, and B. canis*[2]. The disease causes animals to miscarry and become infertile, and it can also be transmitted to humans by touching infected animals or eating infected food, such as dairy products. *B. melitensis*, *B. bovis*, and *B. suis* are three major types of *Brucella* that are prevalent in China; additionally, brucellosis in sheep is most common, followed by *Brucella* in cattle, all of which are the most common sources of brucellosis in humans[3]. Infected animals also develop orchitis, arthritis, and other symptoms. Animals have varying degrees of susceptibility to the disease. In recent years, the number of human and animal infections caused by *Brucella* has been increasing, which seriously threatens people's health and the development of animal husbandry. Over 0.5 million new human brucellosis cases are reported every year worldwide[4,5]. In China, the number of sheep and goat populations infected with *Brucella* is on the rise, and *Brucella* spp. are highly endemic in some areas[6]. Because brucellosis is mostly a chronic disease and there are still some problems with diagnostic methods, it is an important public health problem in many countries, especially in remote rural areas(4). Following *Brucella* infection, treatment is difficult, so the emphasis is on preventing and controlling brucellosis. Currently, the most commonly used vaccines

are conventional live attenuated strains of *B. melitensis* and *B. abortion* B19[7]. However, these vaccines have some disadvantages, such as the possibility of miscarriage in pregnant animals[8]. Additionally, vaccination often complicates the distinction between naturally infected and vaccinated animals, which is not conducive to the diagnosis and control of brucellosis[9]. Therefore, a timely and accurate diagnosis is important for the prevention and control of the disease.

There are many methods available for the diagnosis of brucellosis in animals, such as serological, etiological, and molecular biological diagnostic procedures[10]. Although bacteriological diagnosis is the gold standard, it is not only time consuming and laborious but also can result in operator infection. Molecular biological pathogen detection technology has the characteristics of safety, reliability, high sensitivity, strong specificity, and easy operation. It has begun to be applied in the detection of *Brucella*. The most widely used method is the polymerase chain reaction (PCR) method. Since PCR is a method for detecting nucleic acids, it requires very precise equipment. Generally, expensive equipment prevents this method from being universally popularized. Therefore, it is necessary to design a convenient, rapid and economical diagnosis method[10]. Enzyme-linked immunosorbent assay (ELISA), the rose bengal test (RBT) and the standard tube agglutination test (SAT) can discover the existence of *Brucella* infection by detecting serum antibodies[11]. However, these methods have some disadvantages, such as false-positive and false-negative results. Compared with other diagnostic methods, the outstanding advantages of ELISA are higher sensitivity, specificity, and simple operation. Therefore, it has become one of the most widely used detection methods.

When performing ELISA tests, the choice of antigen is important for achieving high accuracy. Compared with other antigenic molecules of *Brucella*, lipopolysaccharide (LPS) of the smooth *Brucella* genus is the major virulence determinant currently known, and infected animals are prone to produce antibodies against it[12]. Therefore, LPS is the most commonly used antigen in the serological diagnosis of brucellosis. However, serum diagnostic tests for smooth *Brucella* LPS as a diagnostic antigen cannot distinguish infections caused by cross-reactive species; examples are *Yersinia enterocolitica* O9, *Vibriocholerae*, *Escherichia*, and *Salmonella*, which often lead to false positives[13]. Many studies have shown that *Brucella* outer membrane proteins (OMPs), mainly OMP16, periplasmic protein 26 (BP26), OMP2b, and OMP31, have strong immunoreactivity and are suitable for the diagnosis of brucellosis as a substitute for LPS[7,14-16]. False-positive results from cross-reactive antibodies can be reduced by using OMPs to replace previous diagnostic antigens.

Although some previous studies have used *Brucella* OMPs to diagnose brucellosis and achieved good experimental results, there are still many deficiencies that need to be improved. In this research, we used some bioinformatics tools to predict the B- and T-cell epitopes of the four *Brucella* OMPs mentioned above. Then, we designed a recombinant multiepitope protein (rMEP) and evaluated this protein as a serum diagnostic antigen for detecting goat and bovine brucellosis.

Materials And Methods

2.1. Selection of outer membrane proteins

Based on a large number of studies of *Brucella* OMPs, OMPs that have been proven to have antigenicity and exist in different species of *Brucella* were selected as the study proteins. The website https://www.ncbi.nlm.nih.gov/protein/was used to obtain the amino acid sequence of the selected OMPs. To avoid cross-reactions with other bacteria, we used the BLASTP tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to carry out sequence comparison analysis of amino acids and selected the conserved amino acid sequence in *Brucella* according to the comparison results.

2.2. Epitope prediction of outer membrane proteins

ABCpred (http://www.imtech.res.in/raghava/abcpred/)[17,18], Bepipred (http://www.cbs.dtu.dk/services/Bepipred/0)[19], and COBEpro (http://scratch.proteomics.ics.uci. edu/)[20] were used for linear B-cell epitope prediction, and overlapping B-cell epitopes were selected as candidate B epitopes. T-cell epitopes were predicted by T-cell epitope prediction tools at IEDB (http://tools.iedb.org/main/tcell/).

2.3. Construction, expression and purification of rMEP

We used the linker peptide GGGS to tandem candidate B- and T-cell epitopes, inferred codons according to the obtained amino acid sequence, and used the optimization website (http://www.jcat.de/) to perform codon optimization of the prokaryotic expression system. The optimized sequence was transferred to Sangon Biotech (Shanghai, China) for gene synthesis. The coding sequences of six His tags were added to the 3' end of the gene for subsequent purification and identification.

The gene fragment was ligated with the expression vector pGEM-T, and the newly constructed construct was transformed into *E. coli* BL21 (DE3) cells grown in LB medium. The suitable time for us to induce recombinant protein expression was when the optical density values at 600 nm (OD_{600}) reached 0.6. The expression was induced by 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and incubated for 4 h at 37 °C. Bacterial cells were collected by centrifugation and analyzed by SDS-PAGE. The rMEPs were purified by nickel agarose affinity chromatography.

2.4. Serum samples

Ninety-three goat serum samples and 74 bovine serum samples confirmed for brucellosis by both the serum tube agglutination test (SAT) and the Rose Bengal plate agglutination test (RBPT) were used as positive samples. Sixty-six goat and 79 bovine serum samples that were negative for RBPT and SAT were collected as negative samples. All samples were provided by the China Animal Health and Epidemiology Center (Qingdao, China).

2.5. ELISA analysis

The obtained goat and bovine serum samples were detected by indirect ELISA in which rMEP was used as the diagnostic antigen. The purified rMEPs were diluted with PBS buffer solution (0.01 M PBS, pH 7.4) and coated in 96-well immunoassay plates (Corning, USA) at 1 mg per well overnight at 4 °C. Phosphate-buffered saline-Tween 20 (PBST) was added to each well, washed four times, and then blocked with 1% ovalbumin (OVA, TCI, Japan) for 1.5 h at 37 °C, washed four times again and incubated with goat and bovine serum at 37 °C at a dilution of 1:400 for 1 h. The plates were continuously washed and incubated with HRP-conjugated recombinant protein G (dilution of 1:5,000) (Thermo, USA) for 25 min at room temperature. The plates were washed, 100 μL of substrate solution containing TMB (trimethylbenzene) was added to each well, and the plates were placed in the dark at room temperature. After 15 min of color reaction, 2 M H_2SO_4 was added to stop it. Optical density values were obtained at 450 nm (OD_{450}) in an ELISA plate reader (BioTek). All samples were measured twice. We evaluated the effectiveness of rMEP indirect ELISA by comparing PBST and SAT. In addition, to evaluate the detection utility of rMEPs, ELISA was performed again with lipopolysaccharide (LPS, 0.1 mg per well, provided by the China Animal Health and Epidemiology Center) antigen, and the two results were compared and analyzed.

2.6. Sensitivity and specificity

To verify the specificity of the rMEP in the diagnosis of brucellosis, the established iELISA method was used to detect bacteria, including *Yersinia enterocolitica O9*, *Escherichia coli O157:H7*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, and *Salmonella*, in rabbit serum. Brucellosis positive and negative sera were used as controls. Rabbit serum was purchased from Tianjin Biochip Corporation (Tianjin, China). HRP-conjugated goat anti-rabbit IgG (dilution of 1:20,000) (Bioworld, USA) was used. To verify the sensitivity, we selected goat and bovine serum (brucellosis positive and negative controls purchased from APHA, Surrey, UK). All incubated serum dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800 were tested. The OD450 value was read by an ELISA plate reader, and the ratio of the positive serum OD450 value (P: positive) to the negative serum OD450 value (N: negative) was calculated; a positive judgment result was a ratio (P/N)> 2.1, and a negative judgment result was a ratio (P/N)< 2.1. These results indicate whether the method can correctly judge the result and evaluate its sensitivity and specificity.

2.7. Statistical analysis

GraphPad Prism version 6.05 for Windows was used to obtain the dotplot and receiver operating characteristic (ROC) curves.

Results

3.1 Prediction of B- and T-cell epitopes

The amino acid sequences of four outer membrane proteins were obtained from the NCBI database to predict the epitopes of B and T cells. To obtain the most immunogenic B- and T-cell epitopes,

three epitope prediction software programs (ABCPred, BepPred, and COBEPro) and the T-cell epitope prediction tool at IEDB (http://tools.iedb.org/main/tcell/) were applied. Among those epitopes obtained by three epitope prediction software programs, we selected 25 overlapping epitopes as candidate epitopes (see Table S1). BLASTP results also revealed that the selected epitopes are highly conserved in *Brucella*.

3.2. Production and purification of the recombinant protein

Linkers were used to link predicted epitopes to avoid the formation of hinge regions (see Fig. 1). Codon optimization of the prokaryotic expression system was performed according to the amino acid sequence of the constructed fusion protein (see Fig. S1). The synthetic gene was ligated to the expression vector pET-28b, the recombinant vector was transformed into *E. coli* BL21 (DE3) cells in LB medium, and expression was induced in the cells. The induced products were analyzed by SDS-PAGE (see Fig. 2A). Ni-NTA affinity was used to purify the recombinant protein, and the purified protein was analyzed by SDS-PAGE again (see Fig. 2B). The concentration of rMEP protein solution was 1.39 mg/mL in 1 mL.

3.3. Serodiagnostic evaluation of rMEPs by indirect ELISA

To assess the diagnostic potential of the recombinant protein studied in this experiment, 159 goat serum samples were tested using indirect ELISA, including 93 cases of brucellosis and 66 cases of nonbrucellosis. A dotplot summarizes the OD values of the selected samples in this study (see Fig. 3A). The optimal sensitivity and specificity were obtained by ROC analysis (see Fig. 3B). The AUC of this experiment was 0.9976 (95% confidence interval (CI), 0.9930 to 1.002), the optimal cutoff value was 0.5955 (calculated by the Youden index), the diagnostic sensitivity was 96.97% (95% CI, 89.48 ~ 99.63), and the specificity was 98.92% (95% CI, 94.15 ~ 99.97). At this cutoff, 92 of the 93 positive cases were correctly diagnosed as positive, while only 1 was wrongly diagnosed as negative. Sixty-four negative cases were correctly diagnosed as negative, and only 2 were wrongly diagnosed as positive. Compared with those of the SAT and RBPT, the positive and negative diagnostic accuracy of ELISA was 98.92% (92/93) and 96.97% (64/66), respectively. Moreover, we used LPS as the diagnostic antigen of brucellosis for ELISA detection. The obtained data were also analyzed in the same way as rMEP, and a dotplot diagram (see Fig. 3C) and ROC curve (see Fig. 3D) were used to present the results. According to ROC analysis, the AUC was 0.9945 (95% CI, 0.9874 ~ 1.002), the optimal cutoff value was 0.7165, the diagnostic sensitivity was 100% (95% CI, 94.56 ~ 100.0), and the specificity was 95.7% (95% CI, 89.35 ~ 98.82). At this cutoff value, only 4 cases were misdiagnosed as positive.

Furthermore, 153 bovine serum samples were tested using rMEP, including 74 cases of brucellosis and 79 cases of nonbrucellosis. The dotplot summarizes the OD values and optimal sensitivity and specificity obtained by ROC analysis (see Fig. 4A, 4B). The AUC was 0.9974 (95% CI, 0.9938 to 1.001), the optimal cutoff value was 0.7725, the diagnostic sensitivity was 98.65% (95% CI, 92.70 ~ 99.97), and the specificity was 96.20% (95% CI, 89.30 ~ 99.21). At this cutoff, 73 of the 74 positive cases were correctly diagnosed as positive, while only 1 was wrongly diagnosed as negative. Seventy-six were correctly diagnosed as negative, and 3 were wrongly diagnosed as positive. Compared with the SAT and RBPT, the

positive and negative diagnostic accuracy of ELISA was 98.65% (73/74) and 96.20% (76/79), respectively. When LPS was used as the diagnostic antigen, a dotplot diagram (see Fig. 4C) and ROC curve (see Fig. 4D) were used to present the results. According to ROC analysis, the AUC was 0.9869 (95% CI, 0.9727 ~ 1.001), the optimal cutoff value was 0.9695, the diagnostic sensitivity was 95.65% (95% CI, 87.82 ~ 99.09), and the specificity was 97.47% (95% CI, 91.15 ~ 99.69). At this cutoff value, there were 3 cases misdiagnosed as negative and 2 cases misdiagnosed as positive. Finally, we designed a cross table (see Table 1) to show the number of positive and negative samples at different cutoff values.

Table 1. Positive and negative predictive values of the test calculated for different cutoff values.

Cutoff value	Positive		Negative		PPV (%)	NPV (%)
	TP	FN	TN	FP		
0.5955[a]	92	1	64	2	97.87	98.46
0.7165[b]	93	0	62	4	95.88	100.0
0.7725[c]	73	1	76	3	96.05	98.70
0.9695[d]	71	3	77	2	97.26	96.25

TP, true positives; TN, true negatives; FP, false positives; FN, false negatives;

PPV, positive predictive value (TP/(TP + FP)) × 100; NPV, negative predictive value (TN/(TN + FN)) × 100; [a]cutoff value is calculated by rMEP in goats; [b]cutoff value is calculated by LPS in goats; [c]cutoff value is calculated by rMEP in bovines; [d]cutoff value is calculated by LPS in bovines

3.4. Sensitivity and specificity

We selected the serum of rabbits vaccinated with *Yersinia enterocolitica O9*, *Escherichia coli O157:H7*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, and *Salmonella* to verify the specificity of the rMEP protein in the diagnosis of brucellosis. According to the positive result judgment, P/N>2.1, rMEPs had no cross-reaction with the selected serum (Table 2).

Table 2. Specificity results of the indirect ELISA diagnostic method.

Rabbit Sample	OD$_{450}$	P/N
Yersinia enterocolitica O9	0.375	1.053
Escherichia coli O157:H7	0.298	0.837
Vibrio parahaemolyticus	0.404	1.135
Vibrio cholerae	0.359	1.008
Salmonella	0.434	1.219
Negative	0.356	-

To verify the sensitivity of the rMEP in the diagnosis of brucellosis, we also selected goat and bovine brucellosis positive and negative control serum. According to the positive result judgment (P/N>2.1), serum can be differentiated as positive or negative at a dilution of 1:3200 in bovine (P/N=2.34) and 1:6400 in goat (P/N=2.64); additionally, the limit of detection (LOD) was 1:3200 in bovine and 1:6400 in goat (results are shown in Fig. 5).

Discussion

Brucellosis is an easily neglected zoonosis caused by *Brucella*. It is distributed worldwide; in endemic areas, in particular, the establishment of rapid and accurate diagnostic methods is a prerequisite for disease prevention and control. Detection of antibodies using serological methods is often used to diagnose brucellosis in animals. Since B-cell epitopes are molecular sites recognized by antibodies[21], we used a web server and bioinformatics tools to predict the dominant epitopes of *Brucella* outer membrane proteins. Analysis has determined the characteristics of the amino acids that make up the four OMPs, including immunological information analysis and prediction of possible B-cell epitopes, such as hydrophilicity, surface accessibility and β-turn angle[22-24]. However, the success rate of predicting immunogenic B-cell epitopes using a single bioinformatics tool is low. Therefore, we used three different prediction software programs (ABCPred, BepiPred, and COBEPro) and T-cell epitope prediction tools to predict the predominant epitopes with immunogenicity. A variety of OMPs from *Brucella* have been proven to be highly immunoreactive. It has been reported that *Brucella* OMP antigens can be used for serological diagnosis of diseases and overcome the problem of cross-reactivity of LPS-based serological diagnostic methods. Many studies have performed ELISA tests on brucellosis OMP antigens such as OMP10, OMP19, OMP25, OMP28, and OMP31. These studies show that the use of OMPs from *Brucella* cells is very effective for serological diagnosis of the disease. In this research, we selected four OMPs (BP26, OMP16, OMP31 and OMP2b) and obtained 25 candidate epitopes using three bioinformatics tools and a web server.

At present, there are many methods for diagnosing brucellosis in animals, but there are still some disadvantages, such as difficulty in operation and the time commitment. ELISA is faster and more convenient than other methods and has higher sensitivity and specificity, which is consistent with the

goal of this work[25]. In our work, rMEP was designed using the selected superior B- and T-cell epitopes as a diagnostic antigen, and indirect ELISA was used to diagnose diseased and healthy serum. Subsequently, we performed ROC analysis to verify the validity of this test, and we obtained an AUC value of 0.9789. Compared with the LPS antigen, our rMEP has a high accuracy of diagnosis and is almost comparable to distinguishing *Brucella*-diseased animals from healthy animals. rMEP has no cross-reaction with some common foodborne pathogens and has a low LOD, indicating that it has high sensitivity and specificity.

Conclusion

In summary, this study shows that the indirect ELISA detection method based on the newly designed rMEP has high sensitivity and specificity and can be used to diagnose brucellosis in animals. In our study, the *E. coli* expression system was used to overexpress the recombinant protein consisting of four major OMPs from *Brucella* and confirmed that it could diagnose brucellosis in animals. This meets the need to produce a large number of diagnostic antigens in a short time, saves time and avoids the preparation of *Brucella* LPS antigens. In addition, this test method based on rMEP for brucellosis can provide insight into the diagnosis of other infectious diseases. However, the diagnostic method cannot distinguish the type of *Brucella* infection. The diagnostic value of this method for different types of *Brucella* infections is unknown; therefore, further study is needed to differentiate between vaccinated and nonvaccinated animals.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Human and Animal Rights

Not applicable.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Funding

Competing interests

The authors declare no competing interests.

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Figures
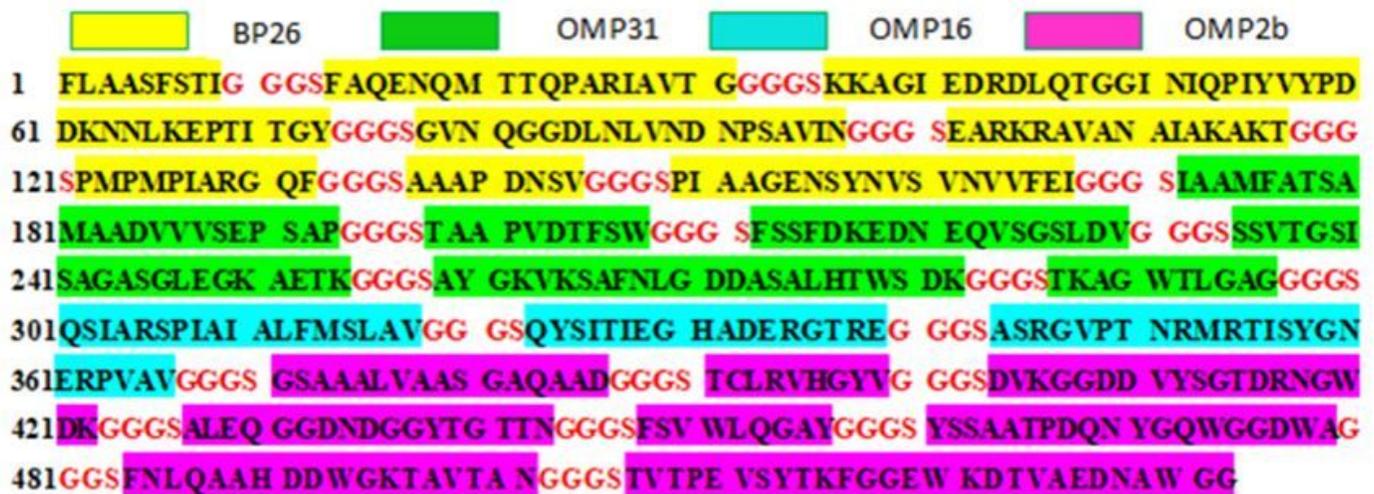
Figure 1

Amino acid sequence of rMEP with "GGGS" as the linker.
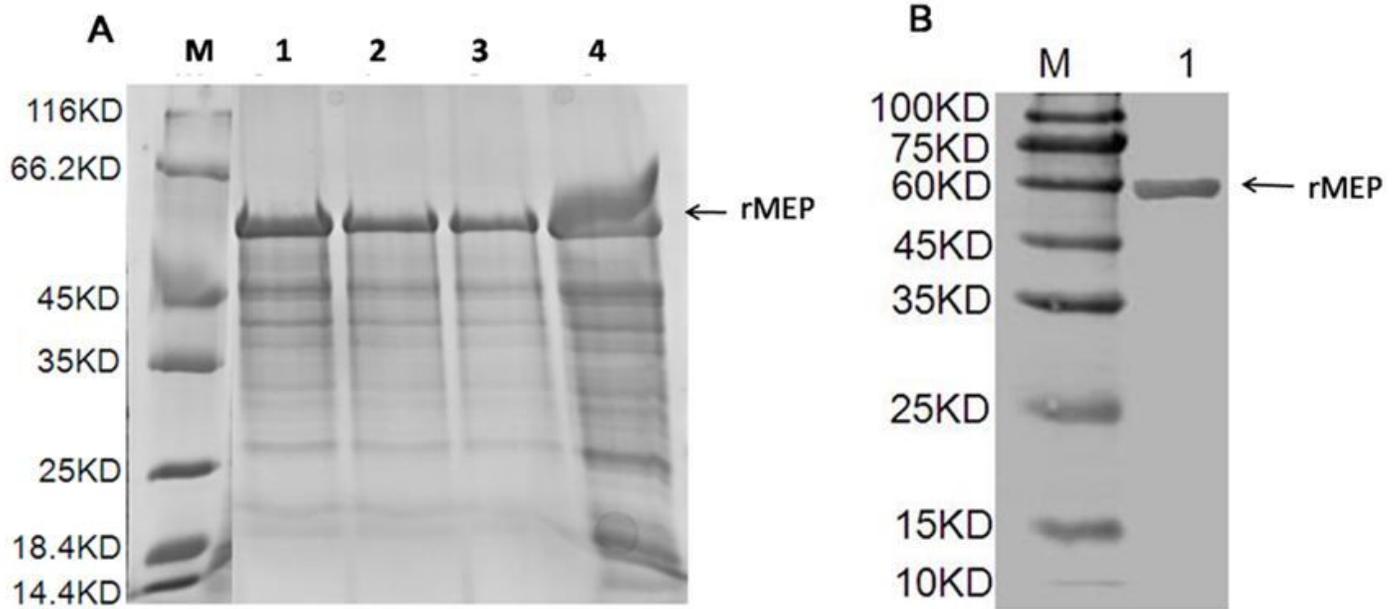
Figure 2

SDS-PAGE analysis of rMEP. (A) Expression analysis of rMEP (M, protein marker; lanes 1-4, elution with imidazole). (B) Purification analysis of rMEPs (M, protein marker; Lane 1, purified recombinant protein).
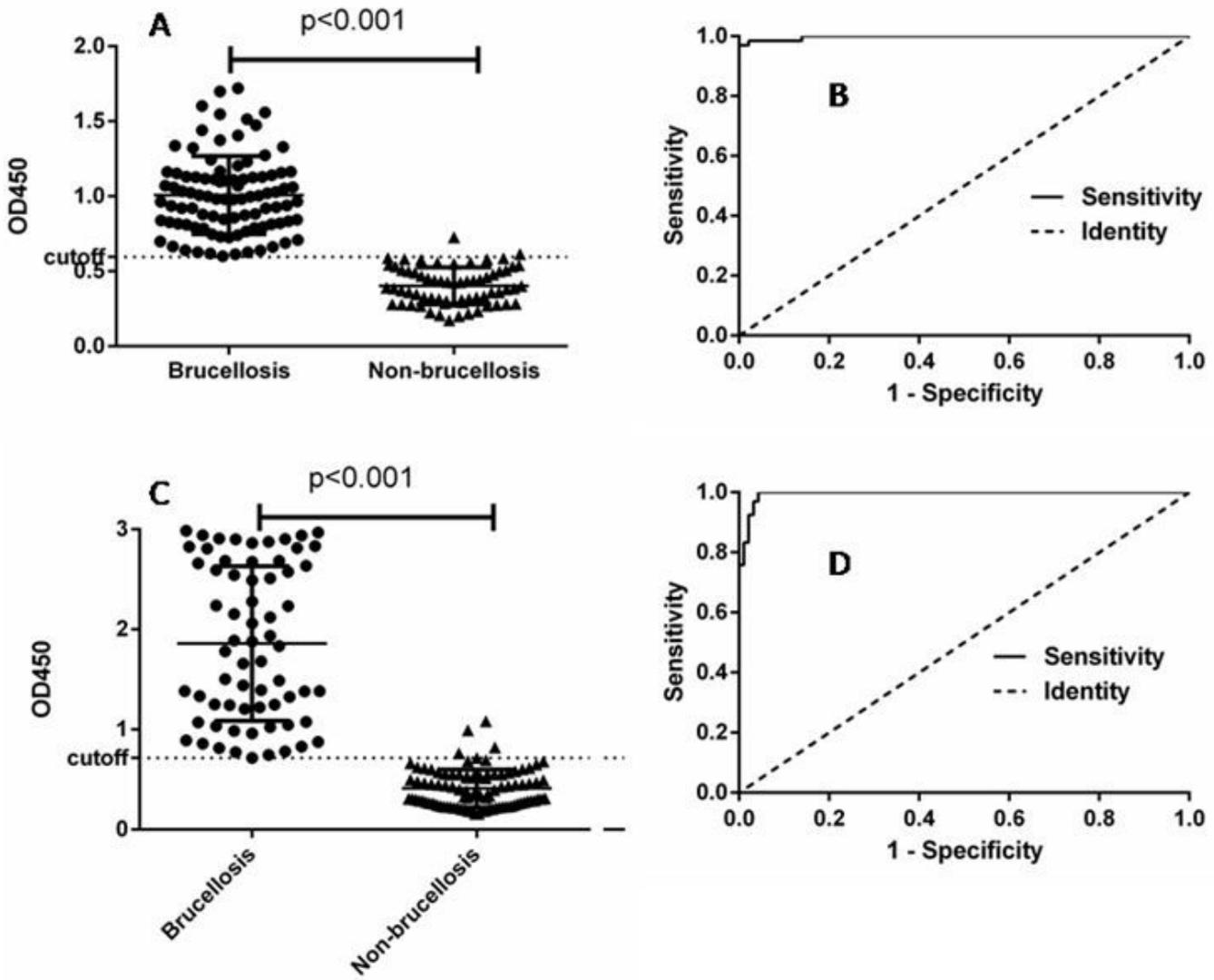
Figure 3

ELISA analysis of goat serum samples. (A) Dotplot of the rMEP ELISA. (B) ROC analysis of rMEP iELISA results. (C) Dotplot of the LPS antigen ELISA. (D) ROC analysis of LPS antigen ELISA results.
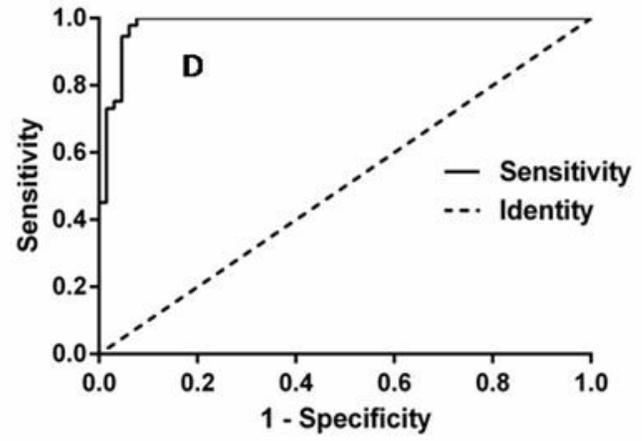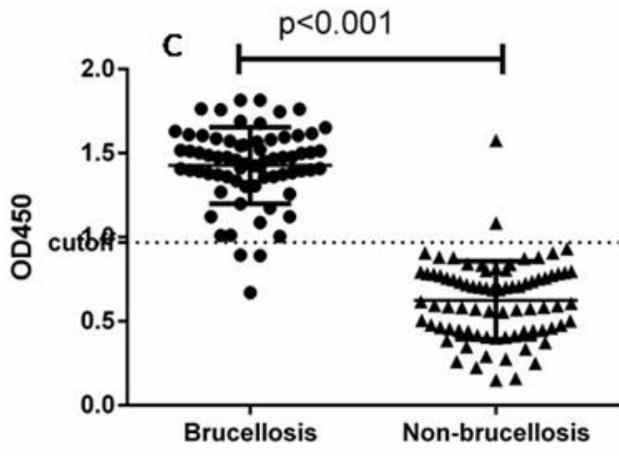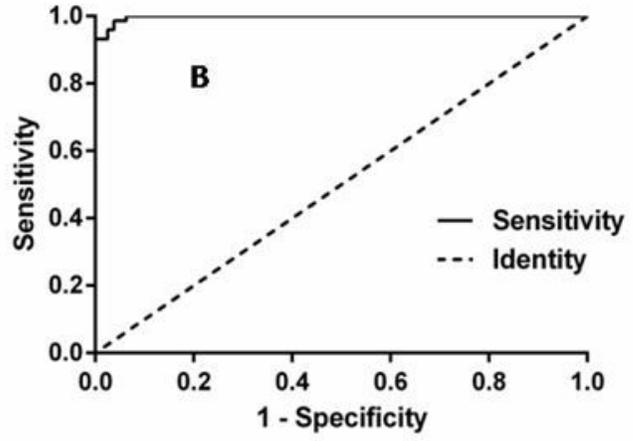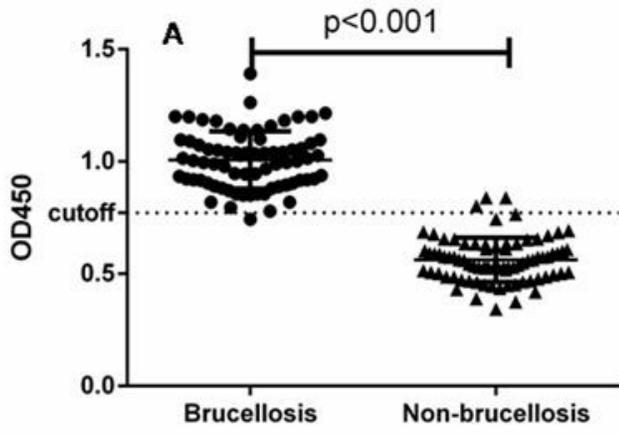
Figure 4

ELISA analysis of bovine serum samples. (A) Dotplot of the rMEP ELISA. (B) ROC analysis of rMEP iELISA results. (C) Dotplot of the LPS antigen ELISA. (D) ROC analysis of LPS antigen ELISA results.
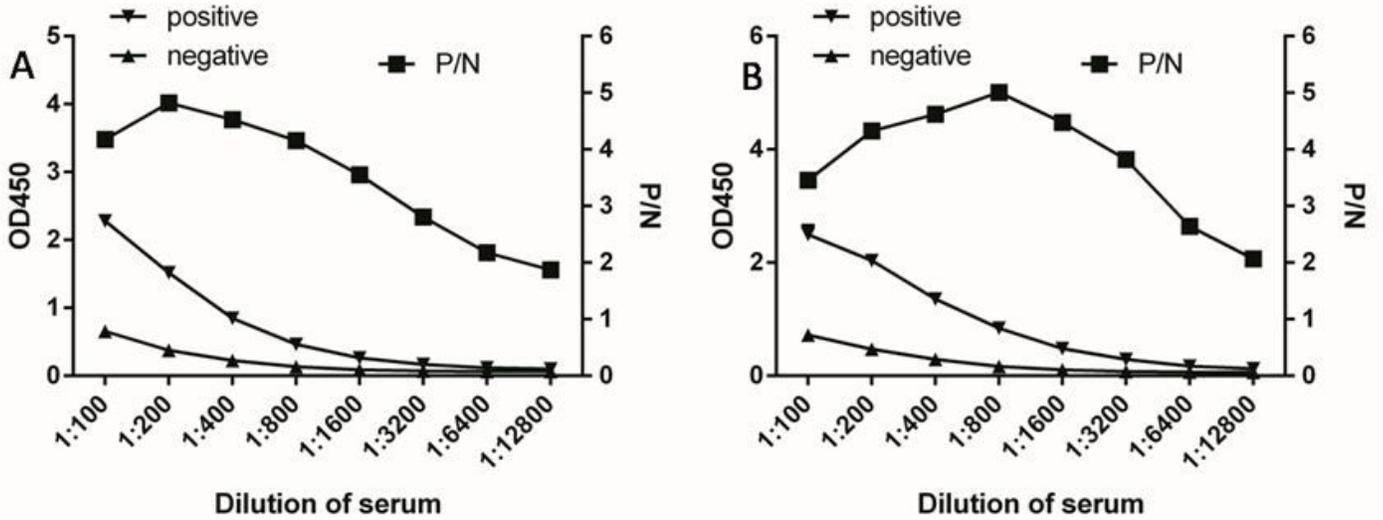
Figure 5

Sensitivity of the rMEP in the diagnosis of brucellosis. (A) bovine serum; (B) goat serum.

Supplementary Files

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