

Evaluation of the Combined Use of Major Outer Membrane Proteins in the Serodiagnosis of Brucellosis

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Background: Brucellosis is a zoonotic disease that causes substantial public health problems and endangers the development of animal husbandry in endemic areas. Early diagnosis of infected animals and humans is a crucial step in reducing the incidence of brucellosis. In this study, we designed different combinations of *Brucella* major outer membrane proteins (omps) including omp10, omp16, omp19, omp25, omp31 and BP26 as antigens and evaluated their efficiency in serodiagnosis for brucellosis. The efficiency assay was conducted using the method of indirect enzyme-linked immunosorbent assay (iELISA) together with a collection of brucellosis-positive sera and healthy sera from multiple species (161 from human, 120 from goat and 144 from cattle). The diagnostic effectiveness of each omp combination was analyzed by receiver operating characteristic (ROC) curve with the software GraphPad Prism version 6.05.

Results: The omp25/omp31/BP26 combination showed the best efficiency in diagnosis for human brucellosis. The area under the ROC curve (AUC) was 0.995 and, compared with the serum tube agglutination test (SAT) and the Rose Bengal plate agglutination test (RBPT), the positive and negative diagnostic accuracies of iELISA were 94.59% (105/111) and 100.0% (50/50), respectively. Evaluation of the 120 goat and 144 cattle serum samples showed that the best combination for diagnosing both omp31/BP26, the AUC was 0.9262 in goat and 0.9344 in cattle, and compared with those of SAT and RBPT, the positive and negative diagnostic accuracies in goat were 72.73% (48/66) and 100.0% (54/54), respectively. The positive and negative diagnostic accuracies in cattle were 79.79% (75/94) and 100.0% (50/50), respectively. Cross-reaction assays showed that omp25/omp31/BP26 and omp31/BP26 do not cross with other common pathogens.

Conclusion: The results indicated that combinations of omps, as protein antigens, can be used to diagnose brucellosis with high accuracy in human, goat and cattle.

Keywords: brucellosis, diagnosis, outer membrane proteins, iELISA

Introduction

Brucellosis is a zoonotic disease with great public health significance. It is caused by *Brucella*, a gram-negative, facultative, intracellular bacteria.¹ The genus *Brucella* includes six classic species namely *B. abortus*, *B. melitensis*, *B. ovis*, *B. neotomae*, *B. suis*, and *B. canis*.² The disease normally causes miscarry and infertile in animals, and can be transmitted to humans by direct contact or consuming infected food, such as dairy products.³ *B. melitensis*, *B. abortus*, and *B. suis* are three major species prevalent in China. Sheep/goats brucellosis is the most common, followed by cattle brucellosis, both are the sources for human brucellosis.⁴ Recently in China, the incidence of human and animal brucellosis has increased, which is seriously threatening the health of people and the development of animal husbandry. To prevent this disease from spreading, a timely and accurate diagnosis is very necessary.

Many methods, such as serological, etiological, and molecular biological diagnostic procedures, are available for the diagnosis of brucellosis in humans and animals. Although bacteriological diagnosis is the gold standard, it is not only time-consuming and laborious but can also result in operator infection. Molecular biotechnology has the characteristics of safety, reliability, high sensitivity, strong specificity, and easy operation and has begun to be applied in the detection of *Brucella*. The most widely used method is polymerase chain reaction (PCR). 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 diagnostic method.⁵ Enzyme-linked immunosorbent assay (ELISA), the Rose Bengal plate test (RBPT) and the standard-tube agglutination test (SAT) can discover the existence of *Brucella* infection by detecting serum antibodies.⁶ 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, ELISA has become one of the most widely used detection methods.

For serological tests, choice of antigen plays an important role in achieving high accuracy. The lipopolysaccharide (LPS) from smooth *Brucella* is the major virulence determinant and also the most commonly used antigen in the serological diagnosis of brucellosis.⁷ However, using LPS as a diagnostic antigen cannot distinguish infections caused by cross-reactive species, such as *Yersinia enterocolitica* O9, *Vibrio cholerae*, *Escherichia*, and *Salmonella*, which often lead to false-positives.⁸ Many studies have shown that *Brucella* outer membrane proteins (omps), including omp10, omp16, omp19, omp25, omp31 and periplasmic protein 26 (BP26), have strong immunogenicity and can substitute for LPS.^{9–12} More importantly, omp antigens can greatly reduce false-positive results caused by cross-reactive bacteria.

Our previous studies have compared the effectiveness of individual omps for the diagnosis of brucellosis; however, there are still works that need to be improved on these omps, especially sensitivity.¹³ In this research, we randomly designed five different combinations among omp10, omp16, omp19, omp25, omp31 and BP26 as antigens, and evaluated their efficiency in human, cattle and goat brucellosis. Hopefully, with these omp combinations, the sensitivity of serological test for brucellosis could be improved.

Materials and Methods

Serum Samples

A total of 111 human, 66 goat and 94 cattle serum samples confirmed by both SAT and RBPT were used as positive samples. Fifty human, 50 cattle, and 54 goat serum samples confirmed by both the SAT and RBPT were used as negative samples. Human brucellosis samples were a gift from the School of Public Health of Jilin University, and human negative sera were provided by the Department of Infection Control, First Hospital of Jilin University. All goat and cattle samples were provided by the China Animal Health and Epidemiology Center (Qingdao, China). All experiments involving human and animal samples were approved by the Ethics Committee and Animal Care and Ethics Committee of Xuzhou Medical University (approval no.: 201801W005). The methods were carried out in accordance with the Declaration of Helsinki and the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Animal Research: Reporting of in vivo Experiments (ARRIVE) guidelines.

Combinations of Outer Membrane Proteins

In our previous study, omp10, omp16, omp19, omp25, omp31 and BP26 have been successfully expressed by a prokaryotic expression system.¹³ In this study, we randomly designed them into five different combinations (Table 1). Information about *Brucella* species and protein accession numbers are listed in Table S1.

ELISA Analysis

The obtained serum samples were tested by our in-house iELISA. Each omp combination consisting of equal concentrations (1.25 µg/mL) of omp protein were diluted in phosphate-buffered saline (PBS) buffer solution (0.01 M PBS, pH 7.4). Ninety-six-well immunoassay plates (Corning, Corning, NY, USA) were then coated with each omp combination at 0.25 µg per well and left at 4 °C overnight. After coating, plates were washed with phosphate-buffered saline-Tween 20

Table 1 Different Combinations of Outer Membrane Proteins

Combination No.	Protein Composition
Combination 1	omp10, omp16, omp19
Combination 2	omp31, BP26
Combination 3	omp25, omp31
Combination 4	omp25, omp31, BP26
Combination 5	omp10, omp16, omp19, omp25, omp31 and BP26

(PBST) four times, and blocked with 5% skimmed milk (Sangon Biotech, Shanghai, China) for 1.5 h at 37 °C. After another washing four times with PBST, 100 μ L human, goat and cattle sera at 1:400 dilution was added to each well and incubated at 37 °C for 1 h. The plates were washed four times and incubated with 100 μ L horseradish peroxidase (HRP)-conjugated recombinant protein G (1:5000 diluted) (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 25 min at room temperature. In the coloring step, plates were washed thoroughly with PBST, then each well was added with 100 μ L of substrate solution containing TMB (trimethylbenzene) placed in the dark at room temperature for 15 min. Finally, 50 μ L 2 M H₂SO₄ was added to each well to stop coloring reaction. Optical density values were obtained at 450 nm (OD₄₅₀) in an ELISA plate reader (BioTek, Winooski, VT, USA). All samples were measured twice, and the average OD₄₅₀ was calculated. In addition, with the same ELISA method, all these sera were tested using LPS (0.1 μ g per well, provided by the China Animal Health and Epidemiology Center) as antigen.

Specificity Assessment

Rabbit sera infected with *Yersinia enterocolitica* O9, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Legionella pneumophila*, *Listeria monocytogenes*, *Vibrio cholerae* and *Salmonella* were used to verify the analytical specificity of the omp combination antigen, according to the established iELISA method. The rabbit serum was purchased from Tianjin Biochip Corporation (Tianjin, China). HRP-conjugated goat anti-rabbit Immunoglobulin G (IgG) (dilution of 1:20,000) (Bioworld, Irving, TX, USA) was used as detecting antibody. All incubated serum dilutions of 1:400 were tested. The OD₄₅₀ value was read by an ELISA plate reader, and the ratio of the positive serum OD₄₅₀ value (S: sample) to the negative serum OD₄₅₀ value (N: negative) was calculated; a positive judgment result was a signal-to-noise ratio (S/N) ≥ 2.1 , and a negative judgment result was a ratio (S/N) < 2.1 . These results indicate whether the method can correctly judge the result and evaluate its analytic specificity.

Statistical Analysis

Dotplot and receiver operating characteristic (ROC) curves were obtained by GraphPad Prism software (version 6.05). The area under the ROC curve (AUC) was collected to judge the effectiveness of omp combination antigens.¹⁴ Other parameters, such as cutoff value, true positives (TP), true negatives (TN), false-positives (FP), false negatives (FN), accuracy, positive predictive value (PPV) and negative predictive value (NPV) were also obtained.²⁰ Cutoff value was calculated by the Youden index (specificity + sensitivity-1). Accuracy, $(TP + TN/TP + FN + TN + FP) \times 100$; PPV, $(TP/TP + FP) \times 100$; NPV, $(TN/TN + FN) \times 100$.¹⁵

Results

Evaluation of Omp Combinations by Indirect ELISA

A total of 161 human serum samples were tested using indirect ELISA, including 111 brucellosis positive and 50 brucellosis-negative sera. The dotplot summarizing the optical density (OD) values of human samples and ROC analysis were showed in Figure 1A and B. The iELISA results showed that the best omp combination was combination 4 (omp25, omp31 and BP26), and the AUC of this combination was 0.9976 (95% confidence interval (CI), 0.9890–1.001). The optimal cutoff value was 0.4756, the diagnostic sensitivity was 94.59% (95% CI, 88.61–97.99), and the specificity was 100.0% (95% CI, 92.89–100.0). At this cutoff, 105 of the 111 positive cases were correctly diagnosed as positive, while only 6 were misdiagnosed as negative. Fifty negative cases were all correctly diagnosed as negative. Compared with the accuracy of the SAT and RBPT, the diagnostic accuracy of iELISA was 96.27% (155/161). Moreover, when using LPS as

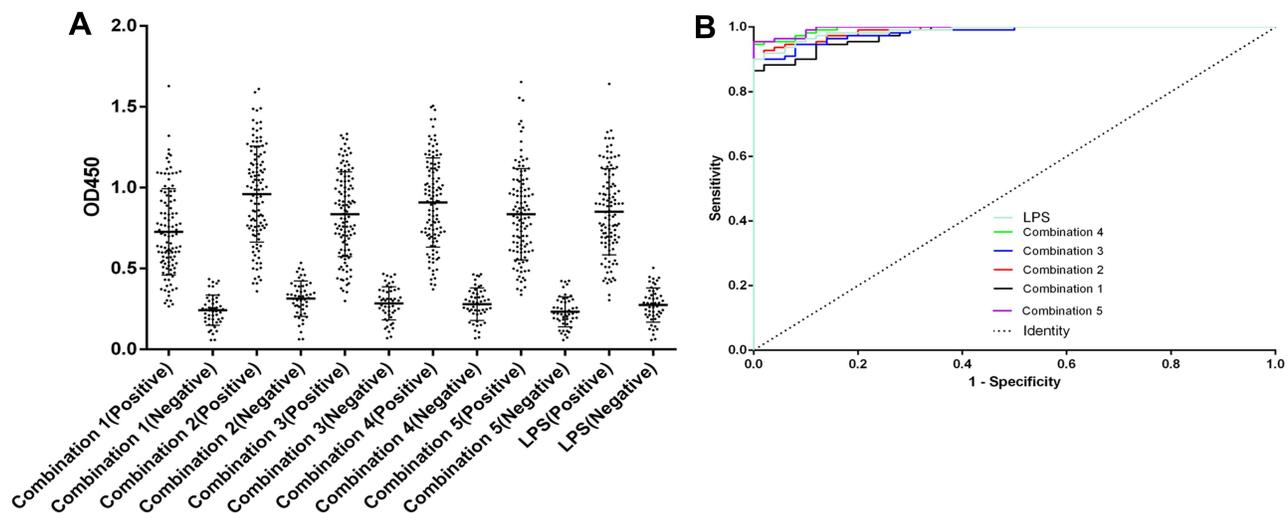


Figure 1 Indirect ELISA analysis of serum samples. **(A)** Dot plot of human sera. **(B)** ROC analysis of human sera.

the diagnostic antigen, the AUC was 0.9881 (95% CI, 0.9768–0.9994), the optimal cutoff value was 0.5066, the diagnostic sensitivity was 90.09% (95% CI, 82.96–94.95) and the specificity was 100.0% (95% CI, 92.89–100.0). At this cutoff value, 11 positive cases were misdiagnosed as negative. The diagnostic accuracy was 93.17% (150/161).

A total of 120 goat serum samples, including 66 brucellosis-positive and 54 brucellosis-negative samples were also tested (see Figure 2A and B). The ELISA results showed that the best combination was combination 2 (omp31 and BP26), and the AUC was 0.9262 (95% CI, 0.8817–0.9707). The optimal cutoff value was 0.7855, the diagnostic sensitivity was 71.21% (95% CI, 58.75–81.70), and the specificity was 100.0% (95% CI, 93.40–100.0). At this cutoff, 47 of the 66 positive samples were correctly diagnosed as positive, while negative samples were all correctly diagnosed. Compared with the accuracy of the SAT and RBPT, the diagnostic accuracy of iELISA was 84.17% (101/120). According to ROC analysis, the AUC of LPS antigen was 0.9374 (95% CI, 0.8973–0.9776), the optimal cutoff value was 0.6198, the diagnostic sensitivity was 77.27% (95% CI, 65.30–86.69), and the specificity was 96.30% (95% CI, 87.25–99.55). At this cutoff value, 15 positive samples and 2 negative samples were misdiagnosed. The diagnostic accuracy was 85.83% (103/120).

Furthermore, 144 cattle serum samples including 94 brucellosis-positive samples and 50 brucellosis-negative samples were also tested (Figure 3A and B). The best combination was also combination 2 (omp31 and BP26), and the AUC was

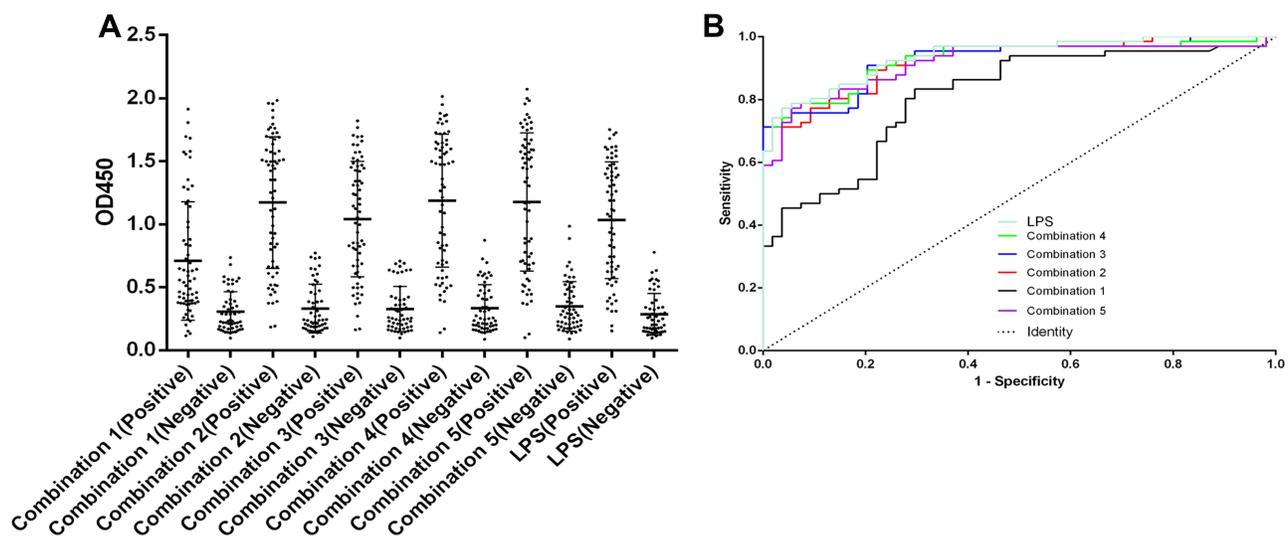


Figure 2 Indirect ELISA analysis of serum samples. **(A)** Dot plot of goat sera. **(B)** ROC analysis of goat sera.

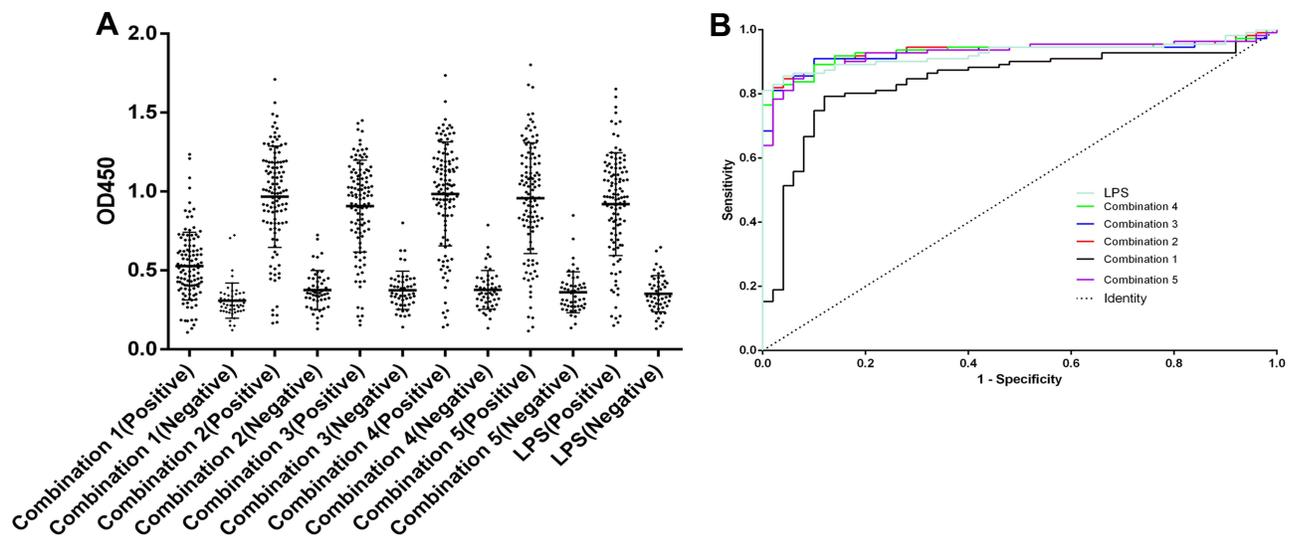


Figure 3 Indirect ELISA analysis of serum samples. (A) Dot plot of cattle sera. (B) ROC analysis of cattle sera.

0.9247 (95% CI, 0.8778–0.9715). The optimal cutoff value was 0.6138, the diagnostic sensitivity was 84.04% (95% CI, 75.05–90.78), and the specificity was 96.00% (95% CI, 86.29–99.51). At this cutoff, 79 of the positive samples and 48 negative samples were correctly diagnosed. Compared with the SAT and RBPT, the diagnostic accuracy of iELISA was 88.19% (127/144). When LPS was used as the diagnostic antigen, the AUC was 0.9164 (95% CI, 0.8679–0.9649), the optimal cutoff value was 0.5397, the diagnostic sensitivity was 85.11% (95% CI, 76.28–91.61), and the specificity was 94.00% (95% CI, 83.45–98.75). At this cutoff value, 14 samples were misdiagnosed as negative, and 3 samples were misdiagnosed as positive. The diagnostic accuracy was also 88.19% (127/144). A cross table was established to show the number of positive and negative samples at different cutoff values (Table 2).

Table 2 Diagnostic Values of the Combinations Calculated for Different Cut-Off Values

Samples	Antigen	Cut-Off value	Positive		Negative		Accuracy (%)	PPV (%)	NPV (%)
			TP	FN	TN	FP			
Human	Combination 1	>0.4271	98	13	49	1	91.30	98.99	79.03
	Combination 2	>0.5026	103	8	49	1	94.41	99.04	85.96
	Combination 3	>0.4739	100	11	50	0	93.17	100.0	81.97
	Combination 4*	>0.4756	105	6	50	0	96.27	100.0	89.29
	Combination 5	>0.4261	105	6	50	0	96.27	100.0	89.29
	LPS	>0.5066	100	11	50	0	93.17	100.0	81.97
Goat	Combination 1	>0.3569	55	11	38	16	77.50	77.46	77.55
	Combination 2*	>0.7855	47	19	54	0	84.17	100.0	73.97
	Combination 3	>0.7119	47	19	54	0	84.17	100.0	73.97
	Combination 4	>0.6280	52	14	51	3	85.83	94.55	78.46
	Combination 5	>0.6724	51	15	51	3	85.00	94.44	77.27
	LPS	>0.6198	51	15	52	2	85.00	96.23	77.61
Bovine	Combination 1	>0.3747	72	22	44	6	80.56	92.31	66.67
	Combination 2*	>0.6138	79	15	48	2	88.19	97.53	76.19
	Combination 3	>0.4700	85	9	45	5	90.28	94.44	83.33
	Combination 4	>0.6478	78	16	49	1	88.19	98.73	75.38
	Combination 5	>0.5398	81	13	47	3	88.89	96.43	78.33
	LPS	>0.5397	80	14	47	3	88.19	96.39	77.05

Note: *The best combination.

Table 3 Specificity Results the Combinations in the Indirect ELISA Diagnostic Method

Antigen	Combination 1		Combination 2		Combination 3		Combination 4		Combination 5	
	OD450	S/N	OD450	S/N	OD450	S/N	OD450	S/N	OD450	S/N
<i>Yersinia enterocolitica</i> O9	0.0667	0.59	0.0682	0.73	0.0629	0.67	0.0611	0.64	0.0701	0.59
<i>E. coli</i> O157:H7	0.0657	0.58	0.0665	0.71	0.0591	0.63	0.0585	0.61	0.0609	0.51
<i>L.P.</i>	0.1495	1.32	0.0935	1.00	0.0921	0.98	0.0994	1.04	0.153	1.29
<i>Salmonella</i>	0.4167	3.68*	0.0929	0.99	0.0984	1.05	0.1028	1.08	0.4157	3.51*
<i>L.M.</i>	0.0704	0.62	0.0729	0.78	0.0691	0.74	0.0691	0.73	0.0702	0.59
<i>V.P.</i>	0.5722	5.05*	0.1389	1.49	0.1328	1.42	0.1256	1.32	0.6017	5.08*
<i>Vibrio cholerae</i>	0.0782	0.69	0.102	1.09	0.1648	1.76	0.066	0.69	0.0683	0.58
Negative	0.1132	–	0.0934	–	0.0936	–	0.0953	–	0.1184	–

Notes: *S/N \geq 2.1, confirmed cross-reaction.

Specificity Assessment

According to the result of iELISA, omp combinations 2, 3 and 4 had no cross-reaction with the rabbits infected with *Yersinia enterocolitica* O9, *Escherichia coli* O157:H7 (*E. coli* O157:H7), *Vibrio parahaemolyticus* (*V.P.*), *Legionella pneumophila* (*L.P.*), *Listeria monocytogenes* (*L.M.*), *Vibrio cholerae* and *Salmonella*. As all the S/N values of these selected sera was <2.1 (Table 3).

Discussion

The omps of *Brucella* are classified and named according to their apparent molecular weight.¹⁶ Omp10, Omp16, and Omp19 are conserved lipoproteins on surface of *Brucella* and the major antigenic proteins. They can induce strong immune responses in mice, and a specific antibody response was also detected in sheep sera.^{17,18} Omp25 is a 25-kD protein and is considered to be crucial to the virulence of *Brucella*.¹⁹ Omp25 can also induce a strong immune response, and monoclonal antibodies against Omp25 have been verified to be useful reagents for the detection of *Brucella* infection in clinical samples.^{20,21} Omp31 is also an important antigenic protein of *Brucella*, which was shown to have good immunogenicity and could elicit strong cellular and humoral immunity in BALB/c mice.^{22,23} BP26 has been identified as an antigenic antigen in infected sheep and humans.^{24,25} The most encouraging result showed that BP26 can differentiate naturally infected sheep serum from vaccinated ones.²⁵ All these omps are highly conserved among the six classical *Brucella* species. Therefore, they might be candidate antigens for brucellosis diagnosis. Many studies have demonstrated that using *Brucella* omps such as omp10, omp16, omp19, omp25, omp28, omp31 and BP26 is very effective way for serological diagnosis of brucellosis.^{26–29} We previously tested human, goat and cattle sera using individual omp as antigen, but the sensitivity of single omp was not as good as the conventional LPS antigen.¹³ We hypothesized that the sensitivity of diagnosis could be improved by using multiple omps. In this research, combinations of omps demonstrated higher accuracy than LPS antigen and almost distinguished all *Brucella*-infected individuals from healthy ones. These results confirmed the hypothesis that the sensitivity of the individual omp can be substantially improved by combining several omp together.

The combination of omp10, omp 19 and omp28 has ever been used to diagnose human brucellosis and combination of omp 22, omp 25 and omp 31 has also been used for the diagnosis of cattle brucellosis.^{28,29} However, there are no studies showing that any combinations of omps can be simultaneously used for human, goat and cattle brucellosis. The study on omp22, omp 25 and omp 31 applied six possible linear B-cell epitopes in tandem and constructed a recombinant protein as diagnostic antigens. However, the accuracy of the recombinant protein is not as good as our full-length omps,²⁸ which may be due to the lack of some important epitopes in recombinant protein. On the other hand, the accuracy of omp combination with omp10, omp 19 and omp28 was 96.0%, which was higher than our combination in diagnosing cattle sera,²⁹ which is probably due to fewer samples used in our study. In the future, we would optimize coating concentration and other conditions which can affect the diagnosis accuracy, and also include more serum samples to verify the diagnostic effectiveness of omp combinations.

The LPS of pathogenic bacteria such as *Yersinia enterocolitica* O9 have severe cross-reactivity with *Brucella*,³⁰ but there is lack of evidence whether omps can also produce cross-reactivity. With commercial rabbit sera, our data confirmed that the omp combinations did not cross-react with *Yersinia enterocolitica* O9 and other foodborne pathogens such as *E. coli* O157:H7, indicating that these protein antigens have high specificity. However, whether the protein antigens have a cross-reaction with the human, goat and cattle samples infected with *Yersinia enterocolitica* O9 and *E. coli* o157:h7 still need to be confirmed by further research.

ELISA is faster and more convenient than other methods and has higher sensitivity and specificity, which is consistent with the goal of this work.³¹ However, this test method based on combinations of omps for brucellosis requires a laboratory and a great deal of equipment, and 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. Additionally, what causes lower positive accuracies in goats and cattle compared to humans is not known. Some sample details, such as the regions from which a sample came, were missing.

Conclusion

In summary, this study showed that the iELISA method based on the newly designed omp combination antigens displayed higher sensitivity and specificity and can be used to diagnose human and animal brucellosis. This study also confirmed that *E. coli* expression system is very useful in producing a large number of diagnostic antigens in a short time, which is faster and safer than LPS antigens production. Finally, whether these omp combinations can differentiate naturally infected animals from the vaccinated ones is still unknown and needs to be further studied by collecting some random samples.

Abbreviations

iELISA, indirect enzyme-linked immunosorbent assay; ROC, receiver operating characteristic; SAT, serum tube agglutination test; RBPT, Rose Bengal plate agglutination test; omps, outer membrane proteins; LPS, lipopolysaccharide.

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Disclosure

The authors declare that they have no competing interests.

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