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Diagnostic Usefulness of Combined Periplasmic Proteins in Bovine Brucellosis

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ABSTRACT

The low efficiency of brucellosis eradication efforts is primarily due to the low performance of conventional serological tests. This is because both traditional and modern tests use the whole cell of the pathogen or its lipopolysaccharides as an antigen, which can lead not only to false-positive results due to antibodies against related bacteria but also to a decrease in the sensitivity of the analysis. The world of veterinary sciences has been actively involved in the search for an immuno-reactive non-polysaccharide antigen specific for *Brucella* spp. that may hold the key to a promising diagnostic test. This study evaluated the serological potential of *Brucella* combined recombinant periplasmic proteins BP26 and superoxide dismutase (SOD) as antigens using indirect enzyme-linked immunosorbent assay ELISA (i-ELISA). The combined antigen was specific for *Brucella* spp., was not recognized by antisera against closely related bacteria, including *Yersinia enterocolitica* O:9 and did not bind to antibodies from negative bovine sera. Blood sera from brucellosis-positive cattle (n=1541), healthy cows (n=608) and brucellosis-free calves (n=46) were used. The efficacy of the combined antigen-based i-ELISA was evaluated by comparing its diagnostic performance with those of the rose Bengal plate test (RBPT), complement fixation test (CFT) and tube agglutination test (TAT). The i-ELISA/BP26+SOD demonstrated the following: sensitivity, 91.6%; specificity, 98.8%; accuracy, 93.6%; positive likelihood ratio, 76.3%; negative likelihood ratio, 0.08%; positive predictive value, 99.5%; and negative predictive value, 82.3%. The kappa coefficient between the serological tests was 0.85, indicating excellent agreement. i-ELISA/BP26 + SOD may be recommended as a supplementary test for brucellosis diagnosis.

Key words: Cattle; Brucellosis; Diagnosis; Recombinant periplasmic proteins; Enzyme-linked immunosorbent assay; Conventional serological tests

INTRODUCTION

Brucellosis, as a widespread zooanthroponosis, causes significant damage to livestock production and is of great social importance. Conservative estimates suggest that more than 300 million of the world's 1.4 billion cattle are infected with *Brucella* (de Figueiredo et al. 2015). It leads to significant economic losses due to the development of reproductive disorders, such as metritis, placentitis, retained placenta, abortions, stillbirth, and abnormal calves in cows, as well as epididymal diseases in bulls. Moreover, diseased cows have reduced milk yields and increased calving intervals (Cárdenas et al. 2019). *Brucella abortus* is the principal pathogen that causes bovine brucellosis; however, species specific to pigs (Ewalt et al. 1997) and sheep (Kahler 2000) may also be pathogenic to cattle. People become infected with brucellosis while caring for a diseased animal, as well as by consuming unpasteurized milk or dairy products (World Health Organization 2006; Khurana et al. 2021). It remains a serious problem in both veterinary and human medicine in Central Asia (Lindahl-Rajala et al. 2017) and Kazakhstan (Baramova et al. 2020).

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One of the main measures in the system of combating brucellosis is the timely isolation of the infected animals. The basic methods for diagnosing brucellosis are bacteriological (i.e., culture isolation) and serological (i.e., detection of specific antibodies to the pathogen). Isolation of *Brucella* is the "gold standard" for definitive proof that an animal is infected. However, not all diseased animals have a positive culture result. Serological methods provide only indirect diagnosis but are the most practical (World Organisation for Animal Health (OIE) 2022). The disadvantages of serodiagnosis include the low sensitivity of conventional tests, weak mutual correlation, and difficulty of interpreting their results (Khurana et al. 2021) as well as the possibility of cross-reactivity of anti-*Brucella* antibodies with antigens of relevant bacteria (i.e., *Yersinia*, *Salmonella*, *Escherichia coli* and so on) (Al Dahouk and Nöckler 2011). The latter drawback is also a characteristic of modern highly sensitive enzyme-linked immunosorbent assays (ELISA), which also diagnose brucellosis by detecting antibodies against bacterial lipopolysaccharides (LPS). This has led to increased interest in alternative antigens, mainly *Brucella* cell wall proteins, which are the most immunogenic substances of the pathogen.

Advances in biotechnology have made it possible to obtain a number of recombinant *Brucella* cell wall proteins and study their serological potential, the results of which have been summarised in our previous review (Bulashev and Eskendirova 2023). In this work, we concluded that recombinant BP26 and SOD can be used as a new antigen for the reliable diagnosis of brucellosis. In recent years, a number of articles have been published on the use of the periplasmic protein BP26 in the diagnosis of *B. melitensis* infection possibly because of its higher risk to humans than other pathogenic species (Kumar et al. 2019; Yin et al. 2020; Bai et al. 2021; Wu et al. 2023). During this period, only a few papers have been published on the immunoreactivity of periplasmic proteins, particularly BP26, in the diagnosis of *B. abortus* infection, although the prospects of using this protein to identify infected cattle have been previously reported. For example, the immunogenic region of BP26 has been used as an antigen in an indirect ELISA (i-ELISA) to differentiate post-vaccination anti-*B. abortus* 19 antibodies in comparison with the rose Bengal plate test (RBPT) (Tiwari et al. 2011). Lim et al. (2012) demonstrated that *B. abortus* 28 kDa outer membrane protein (Omp28, also known as BP26) may be a suitable antigen for developing immunological tests. There were also cautious conclusions about the diagnostic significance of this protein; in a western blot study, BP26 interacted well with *Brucell*a-positive bovine sera, but both whole and truncated protein fragments (i.e, BP26) showed false-positive results against some negative sera (Tian et al. 2020). The diagnostic value of another periplasmic antigen, superoxide dismutase (SOD), is less known than that of BP26. Faria et al. (2020) reported the possibility of the combined use of SOD and malate dehydrogenase (MDH) for the diagnosis of cattle brucellosis and differentiating post-vaccination antibodies from post-infection antibodies; however, both i-ELISA/MDH and i-ELISA/SOD alone failed to distinguish infected from immunised cattle (Andrade et al. 2024).

The increased interest in periplasmic proteins is explained by the fact that they are not only *Brucella*-specific proteins (Nagalingam et al. 2021) but also one of the important virulence factors: Bp26 ensures the binding of the pathogen to extracellular matrix molecules and thereby the spread of the organism within the host (ElTahir et al. 2020), while SOD promotes pathogenicity by protecting the cell from highly toxic oxygen radicals (Pratt et al. 2015). Taken together, the aim of the present work was to study the diagnostic usefulness of a combined antigen consisting of *Brucella* recombinant periplasmic proteins BP26 and SOD for the diagnosis of bovine brucellosis.

MATERIALS AND METHODS

Animal ethics

Ethical approval procedures of the Institutional Animal Ethics Committee's were not carried out as there was no contact with cattle and there were no invasive methods that would harm the animals.

Recombinant periplasmic proteins

Brucella SOD and BP26 were obtained from the corresponding *Escherichia coli* BL21 (DE3) strains as previously described (Manat et al. 2014; Manat et al. 2016). Briefly, amino acid sequences of BP26 and SOD were downloaded from Genbank and compared in a multiple alignment. Solid phase method was used for the synthesis of the target genes de novo by automated DNA synthesizer. The genes encoding periplasmic proteins were synthesized in a PCR using long oligonucleotides as primers. *E. coli* BL21(DE3) were transformed with the plasmid pET-22b(+) carrying the genes of the proteins, and expression of recombinant protein was evaluated. The proteins were purified on fast protein liquid chromatography (Akta purifier 10, GE Healthcare Life Sciences, Cardiff, UK) by metal chromatography using commercial His Trap columns. Inclusion bodies containing the target proteins were harvested by centrifugation, and the supernatant was removed. The residue was dissolved in buffer (20 mM Tris-HCl, pH 8.0, containing 8M urea, 500mM NaCl) and sonicated. Insoluble material was discarded, and the solution was loaded onto a His Trap column and equilibrated with the same buffer. The column was washed with ten volumes of equilibration buffer (20mM Tris-HCl, pH 8.0, containing 8M urea; 500mM NaCl; 20mM imidazole). A linear imidazole gradient (20-500mM) was used for the final elution of the proteins from the chromatographic column. BP26 and SOD fractions were detected at λ=280nm. The purified proteins were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight (mol.w.) of the proteins was determined using the programme for processing electrophoresis results (Vilber Lourmat, Marne la Vallée, France). After electrophoresis, target proteins were detected by immunoblotting using anti-His Tag monoclonal antibodies (Sigma-Aldrich, St. Louis, USA). PageRuler™ Plus Prestained Protein Ladder, 10 to 250kDa was used as marker proteins (Thermo Fisher Scientific, Waltham, USA).

Serum samples

This study used 2195 samples of cattle blood serum, kindly provided by Semey (n=570), Oskemen (n=448),

Karaganda (n=200) and Akmola (n=654) regional branches of the Republican Veterinary Laboratory, Republic of Kazakhstan (RK), and the National Veterinary Reference Center (NVRC), RK (n=323). One thousand five hundred and 41 serum samples were obtained from non-vaccinated cattle kept in brucellosis-affected rural areas. These sera were recognised as true positives based on two-stage serological testing with RBPT and complement fixation test (CFT) with a serum dilution of 1:5 at the first stage and then the tube agglutination test (TAT) and CFT with a serum dilution of 1:10 at the second stage in accordance with the "Veterinary and Veterinary-Sanitary Rules" of the RK. The first stage is used for the primary brucellosis diagnosis. If the results of the RBPT and CFT (1:5) were negative, the animal was considered healthy, and the second stage of testing was not required. This step is mandatory if the results of the first stage are positive or equivocal in one or both tests. The remaining 654 serum samples were collected from unvaccinated cows (n=608) and calves 4-5 months of age (n=46) from various brucellosis-free farms across the Akmola region. These sera showed negative results for *Brucella*-specific antibodies by the battery of conventional serological tests RBPT, TAT and CFTs (hereafter, the combination of these tests will be designated as CSTs - classical serological tests) and were considered true negatives.

Defining parameters for using i-ELISA reagents

The i-ELISA was standardised for the concentration of the combined antigen consisting of equal amounts of BP26 and SOD (BP26 + SOD) and test serum dilution by the checkerboard titration method. Briefly, BP26+SOD was diluted in eight wells of polystyrene carrier (Aptaca, Asti, Italy) in coating buffer (0.05M bicarbonate buffer, pH 9.5), starting at a concentration of 4μg/mL. Further washing and blocking were performed using 10mM phosphate buffered saline (PBS) with 0.05% Tween 20 (PBS-T) and 1% bovine serum albumin (BSA) (Sigma, St. Louis, USA), respectively. Then, brucellosis serum and/or negative serum (Shchelkovo Biofactory, Moscow, Russia) were titrated in the wells in the vertical direction (1:25–1:200), and the plate was incubated for an hour at 4ºC. After washing, the immune complex was detected using rabbit anti-bovine IgG antibody (whole molecule) conjugate (Sigma, St. Louis, USA). Tetramethylbenzidine (TMB) (Immunotech, Moscow, Russia) was used as a colorimetric substrate. Optical density (OD) at 450nm was recorded using a Bio-Rad PR 4100 (Redmond, USA). The established appropriate concentrations of the reagents were used to study cattle blood sera for *Brucella*-specific antibodies.

Serological testing of cattle sera by i-ELISA

Briefly, polystyrene plate wells were sensitized with BP26 + SOD. The plate was washed repeatedly with PBS-T (pH 7.4), blocked with 1% BSA, loaded with an optimal dilution of test and/or control sera, and incubated in a thermostat (1 hour, 37°C). After washing rabbit antibovine IgG conjugate was added, and the plate was maintained at the same temperature. The OD was measured after adding TMB to the wells. ELISA cut-off values were determined by assessing negative calves sera (n=46) results as a mean OD of 450 nm + 3 × standard deviation (SD). All assays were repeated three times.

Determination of the specificity of *Brucella* combined periplasmic antigen

i-ELISA was performed as described above. The specificity of BP26+ SOD was determined using the following commercial homologous i) and heterologous antisera ii): (i) *B. melitensis, B. abortus,* and *B. abortus SAT* positive control sera (APHA, Surrey, UK); dry brucellosis serum and/or dry negative serum (Shchelkovo Biofactory, Moscow, Russia); brucellosis serum and/or negative serum (Antigen, Almaty, Kazakhstan); brucellosis serum and/or negative serum (NVRC, Astana, Kazakhstan); ii) *Yersinia enterocolitica* 09 (BioRad, Marnes-la-Coquette, France); *E. coli* polyvalent diagnostic serum, and *Salmonella* polyvalent O-serum (ECOlab, Elektrogorsk, Russia); diagnostic polyvalent serum for *Shigella flexneri* (Veterinary Research Station [VRS]), St. Petersburg, Russia); diagnostic serum for *Campylobacter fetus* (Agrovet, Moscow, Russia); agglutinating serum for *Leptospira,* serogroup *Icterohemorrhagia* (Armavir Biofactory, Krasnodar, Russia). Rabbit antibovine IgG whole molecule and/or goat anti-rabbit IgG (Sigma, St. Louis, USA) were used as second enzyme-labelled antibodies against homologous and/or heterologous antisera, respectively.

Diagnostic test evaluation

The number of true-positive (TP) and false-negative (FN) cases by i-ELISA/SOD+BP26 in testing CSTs-positive sera and the number of true-negative (TN) and false-positive (FP) cases by the combined antigen-based immunoassay in testing CSTs-negative sera were counted. From these data, sensitivity (Se), specificity (Sp), accuracy (Ac), positive likelihood ratio (PLR), negative likelihood ratio (NLR), positive predictive value (PPV) and negative predictive value (NPV) were calculated using the following equations: Se=TP/(TP+FN); Sp=TN/(TN+FP); Ac=TP + TN/(RBPT, CFT and TAT-positive) + (RBPT, CFT and TAT-negative); PPV=TP/(TP+FP); NPV=TN/(TN+FN); PLR=Se/(100-Sp); and NLR=(100-Se)/Sp (Ismael et al. 2016).

Statistical analysis

A receiver operating characteristic (ROC) curves were used to analyse the immunoassay results (Hanley 1989). The optimal cut-off point for distinguishing between positive and negative samples was determined using the Youden index (sensitivity + specificity minus 1) (Ruopp et al. 2008). The agreement between i-ELISA/BP26+SOD and CSTs was assessed by calculating the kappa coefficient (K).

$$K = \frac{2\times(TP\times TN - FN\times FP)}{(TP+FP)\times(FP+TN)+(TP+FN)\times(FN+TN)}$$

where values of $\kappa>0.81$, $\kappa=0.61$-0.80 and $\kappa<0.41$-0.60 are considered near perfect agreement, substantial agreement, and moderate agreement, respectively. A difference was considered statistically significant at $P\leq0.05$.

RESULTS

Electrophoresis and immunoblotting of *Brucella* periplasmic antigens

The presence of recombinant periplasmic proteins in the transformed cell lysate, after purification by metal affinity chromatography, was detected by SDS-PAGE and immunoblotting (Fig. 1).

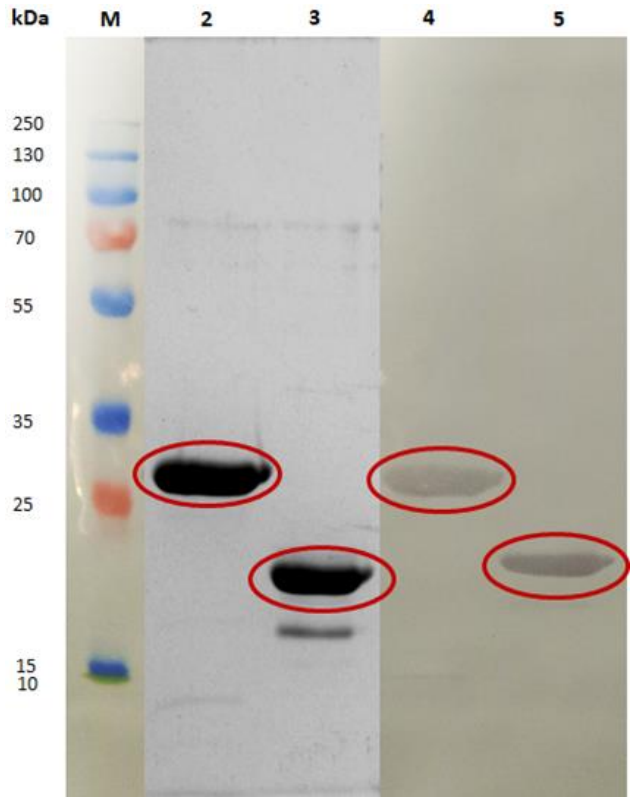

Fig. 1: SDS-PAGE analysis of BP26 (2) and SOD (3) produced by *E. coli* BL21(DE3) and the expressed proteins (4 and 5, respectively) by western blot; M=Markers.

As can be seen from Fig. 1, *Brucella* BP26 and SOD, produced by *E. coli* BL21(DE3), have mol.wt of 27.2 and 20.9kDa, respectively, which correspond to the mol.wt of their natural forms.

The optimal ratio of BP26+SOD concentration and sera dilution for detection of *Brucella*-antibody

Table 1 shows the results of a checkerboard titration to determine the appropriate combined antigen and test serum parameters in i-ELISA.

The most optimal ratio of enzyme immunoassay reagents for serological testing of cattle for brucellosis was observed when using 1.0µg/mL of combined antigen and 1:100 diluted test serum.

Forty-six sera from healthy calves negative for brucellosis were used to determine i-ELISA cut-off value. The mean OD_{450} value of calf serum was 0.177± 0.040, and the cut-off was set at 0.297 [(average OD + 3 × SD (0.177+3×0.040)].

i-ELISA/BP26+SOD on serum samples

After optimising the immunoassay reagents and determining the cut-off value for the combined antigen, 608 *Brucella*-negative sera and/or 1541 *Brucella*-positive sera by CSTs were tested using i-ELISA/BP26+SOD. Sera from 601 healthy cows (98,8%) yielded OD values between 0.0420 and 0.202 (0.177+0.040); however, seven animals (1.2%) yielded ODs exceeding the cut-off value (>0.297; ≤0.898). Samples from 1412 cattle (91.6%) positive for brucellosis by the CSTs gave ODs from 0.528 to 1.686 (1.590+0.312), but 129 (8.4%) animals showed ODs below the cut-off level (0.239+0.043) (Fig. 2a).

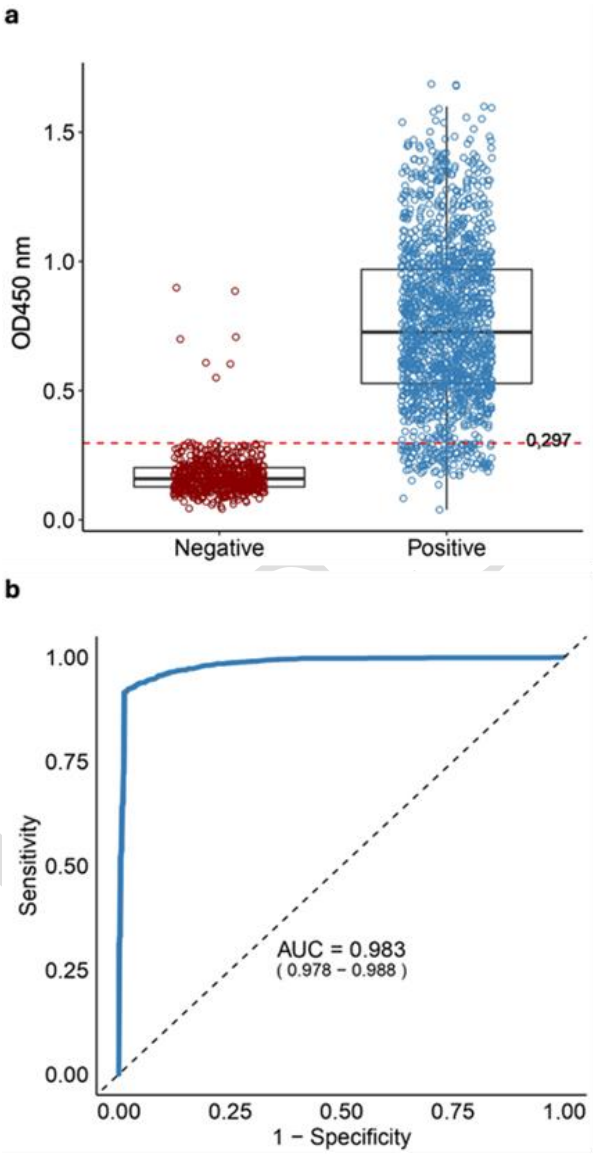

Fig. 2: Dot plot graph analysis of i-ELISA/BP26+SOD results on sera from cattle negative or positive for brucellosis by the CSTs. Each circle shows the OD of a serum from one animal. The cut-off is indicated by the dashed line (a); ROC curve plotted with sensitivity against 1-specificity, AUC - area under the curve (b).

The box plot shows that 54.5% of the positive i-ELISA/BP26+SOD results are located above the median line (OD≥0.726), indicating that more than half of the samples (n=770) have a higher antibody concentration (Fig. 2a). Half of the immunoassay negative results (n=301 or 50.1%) were located below the median line (OD≤0.159), indicating a significant difference between the two groups of animals. The percent positivity values were calculated for the test, resulting in an AUC of 0.983 (0.978–0.988) and a Youden index J of 0.3065 (Fig. 2b).

Diagnostic performance of i-ELISA/BP26+SOD

The efficacy of the combined antigen-based i-ELISA was evaluated by comparing its diagnostic performance with that of CSTs (Table 2).

As shown in Table 2, the i-ELISA/BP26+SOD was characterised by a fairly high specificity (98.8%), i.e. the ability to correctly identify healthy cows in the total number of *Brucella*-negative animals by the CSTs.

Table 1: Determining the optimal ratio of i-ELISA reagents for *Brucella*-antibody detection in cattle sera

Sera dilutions	OD 450 nm	Coating concentration of the combined BP26+SOD antigen (μg/mL)			
		2.0	1.0	0.5	0.25
1:25	P	2.225±0.071	2. 107±0.064	1. 710±0.063	1. 203±0.049
	N	0.371±0.023	0.259±0.027	0.221±0.022	0.184±0.020
	P/N	6.0	8,1	7.7	6.5
1:50	P	2.001±0.055	1.889±0.057	1.446±0.059	0.917±0.031
	N	0.295±0.027	0.163±0.015	0.141±0.019	0.111±0.017
	P/N	6.7	11.5	10.2	8.2
1:100	P	1.613±0.064	1.703±0.045	1.087±0.051	0.705±0.033
	N	0.203±0.012	0.098±0.029	0.073±0.013	0.072±0.001
	P/N	7.9	17.3	14.8	9.7
1:200	P	1.397±0.039	1.248±0.051	0.829±0.052	0.484±0.021
	N	0.162±0.011	0.076±0.012	0.075±0.007	0.061±0.005
	P/N	8.6	16.4	11.0	7.9

P - Positive bovine brucellosis serum OD; N - Negative bovine serum OD.

Table 2: Evaluation of combined antigen-based i-ELISA for the diagnosis of bovine brucellosis as compared to the CSTs

Serological tests used							
CSTs (RBPT+CFT+TAT)				i-ELISA/BP26+SOD			
Brucella-positive sera		*Brucella*-negative sera		TP	FN	TN	FP
1541		608		1412	129	601	7
Diagnostic characteristics of i-ELISA/BP26+SOD							
Se%	Sp%	Ac%	PPV%	NPV%	PLR%	NLR%	K
91.6	98.8	93.6	99.5	82.3	76.3	0.08	0.85

Notes: TP - true-positive, FN - false-negative, TN - true-negative, FP - false-positive, Se - Sensitivity, Sp - specificity, Ac - accuracy, PLR - positive likelihood ratio, NLR - negative likelihood ratio, PPV - positive predictive value, NPV - negative predictive value, K - kappa coefficient.

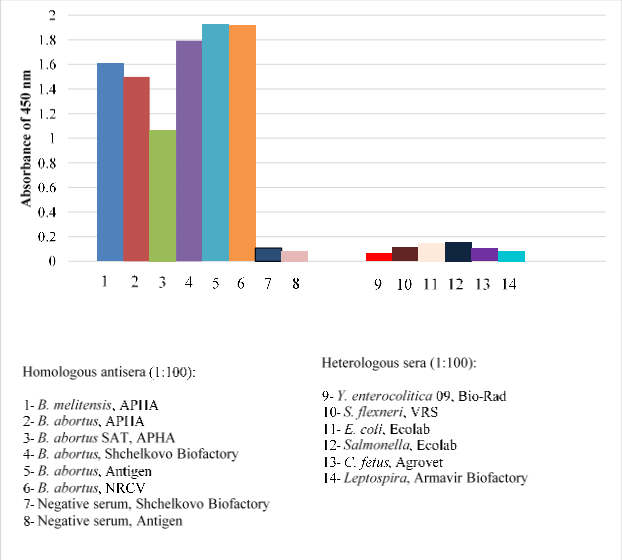

Fig. 3: Specificity of BP26+SOD antigen in i-ELISA

A sensitivity of 91.6% indicates that the result is highly likely to be positive in the presence of infection. These two indicators of diagnostic performance (Sp and Se) provided sufficient accuracy of the immunoassay (93.6%) to recognise infected animals. The PPV (probability of having the infection/disease of interest in a subject with a positive test) and NPV (the likelihood that there is no infection if the test is negative) of the i-ELISA also performed well (99.5 and 82.3%, respectively).

The specificity of the *Brucella* combined recombinant antigen

The specificity of BP26+SOD in the i-ELISA was studied in relation to homologous and heterologous sera (Fig. 3).

As can be seen from Fig. 3, BP26 + SOD showed a well-expressed ability to interact with the antibodies of all homologous antisera used (1–6) at a dilution of 1:100. The average OD values of the liquid in the wells with the combined antigen and antibodies of homologous sera (1.633±0.133) exceeded the cut-off value by an average of 5.5 times (p<0.01). None of the six heterologous antisera (9–14) against closely related Gram-negative bacteria recognised the combined antigen in i-ELISA (OD=0.110±0.014). No reaction was observed between negative bovine sera (7,8) and a cocktail of periplasmic proteins.

DISCUSSION

Animal brucellosis poses major problems for human health, the economics, and international trade (Zhou et al. 2020). The effectiveness of measures to eradicate brucellosis is primarily determined by the accuracy of the diagnosis. No standard assay is currently available to reliably determine the presence and/or absence of anti-*Brucella* antibodies, making it difficult to accurately diagnose the infection. Therefore, a combination of classical tests is required to distinguish healthy individuals from infected individuals in doubtful cases (Tian et al. 2020). Hence, searching for and testing immunogenic antigens is necessary to accurately diagnose *Brucella*-infected cattle, which will significantly reduce the unjustified slaughter of healthy animals due to cross-reactive LPS antibodies (Bulashev et al. 2023).

Brucella SOD and BP26 have become the subject of study, not as much as immunoreactive proteins for improving diagnostic tests but rather as promising substances for the design of modern vaccines. The SOD gene, as part of a DNA or RNA vaccine, induces pronounced immunity in cattle (Sáez et al. 2008) and mice

(Retamal-Díaz et al. 2014). Vector vaccines expressing this periplasmic protein, along with other *Brucella* proteins, can effectively protect laboratory animals from infection (Kim et al. 2016, Bugybayeva et al. 2020). Immunoinformatics analyses (Hashemzadeh et al. 2023) and experimental studies (Wang et al. 2024) conducted on laboratory animals have shown that BP26, as an immunogenic substance, can be used in the construction of combined (subunit) vaccines against brucellosis.

Initial studies investigating the diagnostic value of BP26 for the serodiagnosis of ovine and human brucellosis were promising (Cloeckaert et al. 2001; Seco-Mediavilla et al. 2003; Gupta et al. 2010; Thavaselvam et al. 2010; Koyuncu et al. 2018); however, some refinements were later made. It has been reported that the specificity of ELISA/BP26 depends not only on the pathogen species but also on the host as well as the epizootic situation in the region. Only sheep and goats infected with *B. melitensis*, and goats infected with *B. abortus* had antibodies specific to BP26; however, *B. abortus*-infected cattle and sheep cannot react with this protein (Xin et al. 2013). A species-specific immune response to BP26 (Omp28) was previously established by Lindler et al. (1996), who reported the absence of an antibody response to this protein in cattle and pigs naturally infected with brucellosis, but it was highly antigenic in rodents and goats. Letesson et al. (1997) explained this phenomenon by the endemicity of the disease in the regions where the blood sera were collected. In their opinion, these proteins are less antigenic for animals from brucellosis-free regions than for those kept in brucellosis-endemic zones. Regardless of the explanation for this phenomenon, periplasmic proteins are likely to have a diagnostic value for screening animals from endemic regions. Guo et al. (2024) described the efficacy of competitive ELISA based on BP26 specific monoclonal antibodies for differentiating infected from vaccinated animals diagnosis in China, where BP26-delited vaccine is recommended for brucellosis diagnosis. We have previously demonstrated the superiority of BP26 and/or SOD over Omps when used separately as antigens for testing cattle in brucellosis-endemic regions using i-ELISA (Bulashev et al. 2019). Currently, a promising approach to improving the serodiagnosis of brucellosis is the selection of a combination of pathogen-specific proteins as antigens for serological tests. The BP26 (Omp28) combination with Omp10 and 19 showed higher antigenicity than did individual Omps in TAT-positive cattle sera (Simborio et al. 2015) and BP26 + Omp31 was the best cocktail for screening cattle and goats for brucellosis (Yao et al. 2022).

In this study, we determined the diagnostic value of an i-ELISA based on a cocktail of BP26 and SOD and compared it to that of CSTs. They contain approximately the same mol. w. as their natural analogues (26 and 20kDa, respectively) (Bricker et al. 1990; Rossetti et al. 1996). Minor differences within one kDa are most likely due to the use of different methods to determine mol. w. The results of determining the optimal ratio of i-ELISA reagents showed that for serological testing of cattle for brucellosis, the wells should be coated with a combined antigen at a concentration of 1.0μg/mL, and blood serum should be used at a dilution of 1:100 with PBS-T. With these parameters, the average OD of *Brucella*-positive serum exceeded that of negative serum by 17.3 times (Table 1).

The combined antigens were unique for *Brucella* spp. and did not bind to the antisera used against closely related bacteria, including *Y. enterocolitica* O:9, or to negative bovine sera. For example, the antibodies of all six commercial *Brucella*-positive sera strongly bound to the combined antigen, showing an OD ranging from 1.066 to 1.914, while the extinction index of wells sensitized with heterologous bacteria did not exceed 0.155 (Fig. 3).

This is the first study to demonstrate the absence of similar epitopes between the periplasmic proteins of *Brucella* spp. and Gram-negative bacteria from the genera *Shigella*, *Campylobacter*, and *Leptospira* (Fig. 3). The specificity of two separate proteins BP26 and SOD for *Brucella* was also demonstrated by Nagalingam et al. (2021) using rabbit anti-*Yersinia* serum in western blots. Moreover, Seco-Mediavilla et al. (2003) found that BP26 is highly conserved in the genus *Brucella*. We later reached a similar conclusion regarding SOD (Manat et al. 2014).

The usefulness of the combined antigen was evaluated and compared with the CSTs of 2195 bovine sera. The sensitivity of the i-ELISA/BP26+SOD was quite high (91.6%). Here, taking into account the specificity of the combined antigen to *Brucella* spp., it can be assumed that positive results of the CSTs for brucellosis in 129, or 8.4%, of i-ELISA-negative cattle may be caused by cross-reactive antibodies. In a study of the CSTs-negative sera, antibodies against BP26+SOD were detected in 7 (1.2%) animals, which yielded an i-ELISA specificity of 98.8% (Table 2). Although these sera were obtained from unvaccinated cows in brucellosis-free rural area, we cannot exclude the possibility of movement of individual animals from neighbouring areas where brucellosis is being eradicated using a vaccine. Here we cannot but exclude the possibility of detecting specific antibodies in the sera of healthy animals due to the phenomenon of immunizing subinfection, in which microbes that enter the body die, causing only a specific reaction and promoting the formation of immunity. In this case, no functional disorders occur, and the animal is not a source of the pathogen (Sochnev et al. 2021). Overall, the accuracy of i-ELISA/BP26+SOD, defined as the ratio of TP + TN to CSTs-positive + CSTs-negative, reached 93.6% (Table 2).

The analysis of immunoassay results highlights its diagnostic efficacy in distinguishing between brucellosis-negative and -positive cattle (Fig. 2a). The ROC curve analysis further validates its performance, revealing a high AUC of 0.983 (95% CI: 0.978–0.988), which indicates strong sensitivity and specificity. The calculated Youden index J of 0.3065 reinforces the test's robustness, underscoring its potential utility for accurate brucellosis detection in cattle populations (Fig. 2b).

PLR and NLR are the best indicators of the efficacy of immunological tests. These ratios allowed us to compare the likelihood of obtaining a positive result if the animal truly had the disease with the corresponding likelihood if it did not. Because PLR and NLR effectively summarise the information contained in the four parameters (Se, Sp, PPV, and NPV), they are considered more valuable to clinicians (Bruno 2011). The PPV and NPV of i-ELISA showed good results (99.5 and 82.3%, respectively). This demonstrates the high reliability of the test in correctly identifying the presence or absence of infection. The higher the PLR, the greater the confidence that a positive test indicates the

animal has a disease. Its value of 76.3% in our study indicates a good chance that the test results are true (Table 2). The NLR shows the relationship between the likelihood of a negative test result in the presence of disease and the likelihood of a negative test result in the absence of disease, and the value of 0.08 indicates that a negative result reliably excludes the disease. It is usually less than 1.0, and the lower the NLR, the greater the confidence that a negative test indicates true absence of disease. The kappa coefficient of 0.85 between i-ELISA/BP26+SOD and CSTs results indicates excellent agreement.

Conclusion

To the best of our knowledge, this is the first study to investigate the diagnostic value of the periplasmic proteins cocktail (i.e., BP26+SOD) in brucellosis. BP26 and SOD, as conserved, immunoreactive, and specific proteins for the genus *Brucella*, in combination, provide a reliable serological diagnosis in cattle. i-ELISA/BP26+SOD may be useful as a supplementary tool for current serological tests for brucellosis diagnosis. However, further studies using PCR and/or culture isolation are needed to prove that combined periplasmic antigen-based immunoassays are more reliable than traditional serological tests.

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