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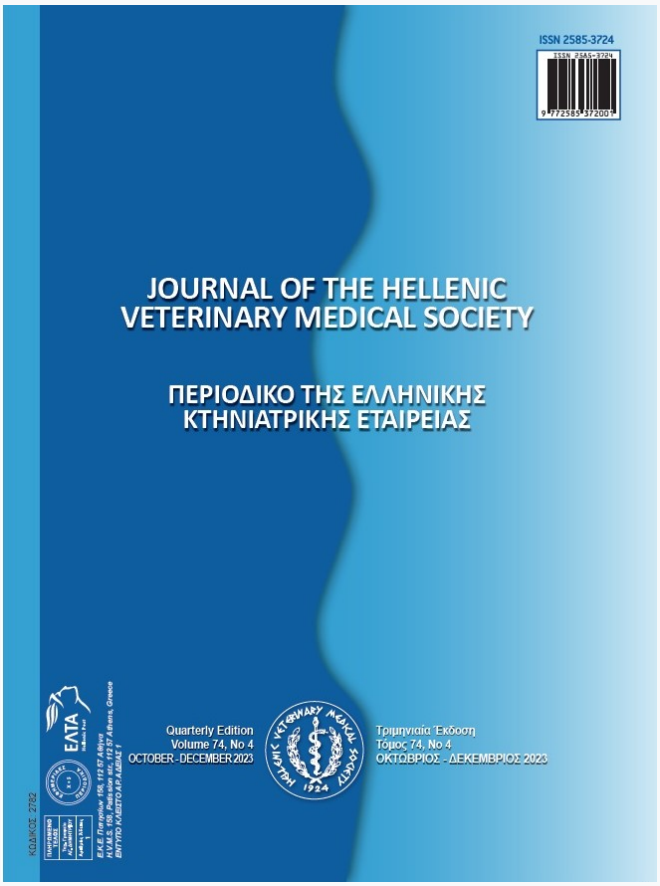

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Research article
Ερευνητικό άρθρο

Serologic and bioinformatics analysis of the Brucella cross reacting antigens

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ABSTRACT: *Brucella* species as zoonotic agents could infect humans and various species of animals. The accurate diagnosis of the infected animals could limit the prevalence of the disease. The current study aimed to identify the cross-reacting antigens among *Brucella* and some of the bacteria that possess antigens of this sort. In addition, the role of the cross-reacting antibodies in the serologic detection of *Brucella* was investigated by comparing the indirect and absorbed indirect ELISA results. Total of 36 sheep serum samples were enrolled in the current research. Antibody titers against *Brucella abortus*, *Salmonella typhimurium*, *Pasteurella multocida*, *Yersinia enterocolitica*, *Proteus vulgaris* and *Escherichia coli* were determined using the indirect and absorbed indirect ELISA. The absorbed ELISA was developed by reacting of the serum samples with the mentioned bacteria; after that, the treated serum samples were tested against *brucella* using indirect ELISA. The cross-reactivity between *Brucella* and other bacterial antigens were defined using the bioinformatics and western-blotting assay. The positive samples have significant IgG antibody titer against all the tested bacterial strains. Absorption of the cross-reactive antibody significantly reduced the ELISA optical density ($P \leq 0.05$) against *Brucella*. Various proteins with different molecular weights were detected in other bacteria that reacted with positive serums samples using immunoblot assays. Also, there was a significant correlation between sequence and structural identity ($P \leq 0.001$). Two proteins including Cu/Zn superoxide dismutase and methionine aminopeptidase of the mentioned bacteria had significantly higher similarity scores to *Brucella* proteins ($P \leq 0.001$). Different IgM and IgG antibodies titer against other bacteria were existed in Brucella-positive samples; additionally, the anti-Brucella antibody in each of the positive samples had different cross-reactivity against antigens of the other bacteria.

Keywords: *Brucella*; Cross reaction; Antibodies; Bacteria; Sheep

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INTRODUCTION

Brucella species gives rise to zoonotic diseases that spread either through a direct contact or animal products (Hans et al., 2020). Brucellosis could affect various species including humans, cattle, sheep, goat, buffalo, pig, rodents and marines. The accurate diagnosis of brucellosis could curb the prevalence of the disease. The infection causes unspecific clinical symptoms (Buzgan et al., 2010); therefore, diagnostic tests have the main role in the detection of the infected individuals. Different serological, bacteriological and molecular methods are used for the detection of the infection in animals (Nielsen and Yu, 2010; Godfroid et al., 2010; Poester et al., 2010).

Serological tests have an edge over the bacteriological and genomic detection of the brucellosis; that is, these tests are always used for screening and the final diagnosis of the disease due to their simple protocols, reproducible results, and being cost-effective. Genomic and bacterial culture tests may produce false negative results especially for chronic brucellosis or in treated individuals. Also, the accuracy of the culture tests depends on the circulating bacterial number, species of the *Brucella*, type of the culture medium and the technique employed. Cross-reacting antibodies remain as the main causes of the high false positive results of the serologic test (Hans et al., 2020). The *Brucella* antigens have similarity in terms of structure and sequence to other bacteria, especially the gram-negative bacteria such as *Escherichia coli H7:O157*, *Yersinia errterocolitica*, *Salmonella typhimurium*, *Vibrio cholera*, *Francisella tularensis*, *Pasteurella* species, and *Proteus vulgaris*; these similarities may becaused by the cross reaction of the produced antibodies that result in false positive outcomes in serologic tests (Corbel, 1985; Bounaadja et al., 2009; Nielsen & Yu, 2010; Patra et al., 2014; Hans et al., 2020).

The induction of the IgM titer after encountering the cross-reacting antigens leads to false results especially in agglutination-based tests (Nielsen et al.,1984; Shemesh &Yagupsky, 2011). The consequence of these cross reactions manifests the control program of the infection and resulted in economic losses. Due to higher sensitivity and specificity, ELISA is preferred for the detection of the infected individuals to other serological tests (Hans et al., 2020; Lotfi et al., 2022). *Brucella abortus* have smooth lipopolysaccharide (SLPS) which contain a lipid Athat anchors to the cell wall, the intermediate core region, and an immunodominant O-polysaccharide (OPS) which have common epitopes in all of the smooth species (Edmonds et al., 1999). So, the bacterial whole cells, SLPS or OPS are always used for the serological detection of the animals infected with all the smooth species (OIE, 2021_b).

The commercial kits based on indirect enzyme immunoassays could provide high sensitivity; however, due to the cross reacting feature of the antibody with other bacteria and *B. abortus* S19 vaccinal strain, the method does not yield faultless specificity. Numerous antigens have been used to mitigate the noted problems; despite some success, cross reacting antibody remained as the chief problem in the precise detection of the *Brucella* (Mahajan et al., 2005; Robles et al., 2009). Moreover, animals have variable immune responses against individual antigens; a weak and strong immune response causes false negative results in ELISA and agglutination tests, respectively. Besides, older animals always have prolonged high levels of the antibodies titer against cross-reacting endemic microorganisms.

The current study aimed to identify the sequence and structure similarity between *Brucella* and some of the reported bacteria using bioinformatics analysis; determining the cross-reacting antigens, using the western-blotting assay is another aim of the current study. Also, an indirect and developed absorbed in-direct ELISA were performed to evaluate the cross reacting antibodies.

MATERIALS AND METHODS

Sampling

This study used a total of 36 sheep serum samples positive or negative results for the Rose Bengal test consisting of male and female animals in herbs of Khuzestan province, Iran. The selected animals aged more than 2 years. The animals had no history of vaccination or abortion in the last year. Blood samples of 10 mL were obtained using a sterile vacutainer tube from the jugular veins of the animals without any anticoagulant element. The serums were separated using centrifugation at 3000 rpm for 10 minutes. The induction of humoral immune response was evaluated by using Rose Bengal (Razi Vaccine and Serum Research Institute, Iran). The serological tests were performed according to OIE terrestrial manual (OIE, 2021_b). 30 μl of serum samples was pipetted onto a clean slide and an equal volume of rose Bengal antigen was added. After mixing, the agglutination reac-

tion was recorded within 4 minutes.

Bacterial strain

The isolates of *Brucella abortus* (*Iriba Vaccine), Salmonella typhimurium* (PTCC 1709), *Pasteurella multocida* (ATCC 12945), *Yersinia enterocolitica* (PTCC 1151), *Proteus vulgaris* (PTCC 1861) and *Escherichia coli* (PTCC 1399) were prepared from the archive section of the microbiology department of the veterinary medicine. The bacteria were cultured in blood agar medium and incubated at 37 ° C for a period of 24-72 hours depending on the type of bacteria. The biochemical tests were used to confirm the identity of the purred bacteria. The obtained bacterial samples were centrifuged and washed 2 times using phosphate buffered saline (PBS). After that the bacteria were killed by incubation on PBS which contained 0.5% formaldehyde; to ensure the inactivation the inactivated bacteria were cultured on a blood agar medium. The killed bacteria were washed three times and sonicated for 10 minutes. The final concentrations were adjusted to an optical density equal to 0.12 nm using a spectrophotometer.

Indirect and absorbed indirect ELISA

To determine the relative reactivity of the positive serum samples, the entire cell antigens of the mentioned bacterial strains were used. Indirect ELISA was done by coating the micro-titer plates wells (Biofile, China) with 100 µL of sonicated whole cell bacterial antigens 10 µg/mL which was diluted in 0.1 M of carbonate buffer (pH 9.6); subsequently, the plates were incubated at 4 ºC overnight. The plates were then washed with 0.1 M PBS pH 7.2 containing 0.05% Tween-20 (PBS-Tween) and 200 µL of 4% skimmed milk in PBS-Tween and then added to the wells and incubated at 37 ºC for 2 hours. The wells were washed again with PBS-Tween, 100 µL aliquot of serum samples was diluted 1:100 in PBS and added to each well. The plates were incubated at 37 ºC for 1 hour. Wells were washed as before and incubated with Horseradish peroxidase-conjugated protein A&G (1:5,000 dilutions). The plates were incubated for 1 hour, washed as described above and each well was treated in the dark for 10 minutes with 75 µL of TMB substrate. The reaction was terminated by adding 75 µL of 2 M solution of sulfuric acid; likewise, the optical density (OD) at 450 nm was measured in an ELISA reader (AccuReader, Taiwan). To ensure the accuracy of the ELISA assay, the negative control sera (negative serum for *Brucella* and PBS) was included in each plate. Each serum was tested in duplicate and the data were recorded as mean OD.

Absorbed indirect ELISA was done in the same manner with the difference that the serum samples were incubated for 1 hour in coated wells with bacteria other than *Brucella*. The serum samples were pipetted out from each well carefully and underwent the next incubation in coated wells with *Brucella* antigens. The remaining steps were similar to those described for indirect ELISA.The ELISA results were considered positive when the optical density was greater than the mean O.D of the negative samples+ 3×standard deviations of the negative control samples (in triplicate).

Western blot assay

The Western blot assay was performed using the electrophoresis of the sonicated bacteria and protein ladder (Sinaclone; CAT. No: SL7001). The prestained protein molecular weight marker contained 235, 170, 130, 93, 70, 62, 53, 41, 30, 22, 18, 14 and 9 kDa proteins. Briefly, electrophoresis was performed with a vertical electrophoresis tank utilizing the Laemmli discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 11% of the separating and 4% of the stacking polyacrylamide gel. The samples were mixed in a 62.5 mM Tris-HCl sample buffer (pH 6.8) containing 2.5% SDS and 0.1 M 2ME. An aliquot of the sample solution was boiled for 10 minutes, and then 25 µL was loaded onto each well. The gel was run at a constant voltage (100 V) for 90 min, using the running buffer (25 Mm Tris base pH 6.8, 192 mM glycine, 1% SDS). The polyacrylamide gels were stained for 30 minutes with Coomassie staining solution followed by de-staining with 7% acetic acid solution overnight; the same gel were transferred to PVDF membrane after electrophoresis (Elabscience; Cat. No: E-BC-R266) for western blot analysis. The transfer was performed for 2 hours with 60V in a Mini Trans- Blot Electrophoretic Transfer Cell (Bio-Rad). Following the transfer, the membrane was blocked with a 5% skimmed milk buffer for 2 hours followed by three washing with PBS-T. The membrane lanes containing the bacterial antigens were incubated for 1 hours at 37 ºC with a 1:20 dilution of the test serum. Subsequently, the membrane was washed as before and incubated with HRP-conjugated protein A&G (Raha Padtan Azma, Iran,) at a dilution of 1:1000 for 60 min at room temperature. After washing, the substrate 4-chloro-1- naphthol (Sigma) was added for color development; after 15

minutes, the membrane was washed using the double sterilized water for 3 times.

Bioinformatics analysis

The protein sequences of *Brucella* and all of the other bacteria were retrieved from NCBI database. The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) genome databases provides free access to available biomedical and genomic information of vertebrates and other eukaryotic species. Swiss model tools were used for the prediction of the three-dimensional structure of proteins. SWISS-MODEL is a web-server for predicting the homology modeling of protein 3D structures (http://swissmodel.expasy.org/). Then, energy minimizing has been done for each 3d predicted structure by SPDBV (Swiss-PdbViewer- 1.4.0 version). Sequence and structure comparisons with other bacteria were performed by BLAST on NCBI, Softberry and 3D-Match tools, respectively. The % sequences and structures identity, Zscore and root-mean-square deviation (RMSD) values were extracted and analyzed statistically. The acceptable three dimensional similarities should have a R.M.S.D value below 1.5 or 2 Å based on the ligand size (Hevener et al., 2009). The Zscore values greater than 3.5 discovered the statistical significance of the structural alignment between the compared proteins (http://linux1.softberry.com/berry.phtml?topic=3dmatch&group=programs&subgroup=3dexpl). Pictures of 3D structures have been taken by the Chimera software (1.11.2 version).

Statistical analysis

The descriptive and analytical evaluation of the data was performed using the SPSS software version 16.0 (Statistical Package for Social Sciences, Chicago, IL, USA). Data analysis was performed by Pearson's correlation coefficient, paired samples t test and repeated measures ANOVA. Also, the differences were considered statistically significant ($P \leq 0.05$).

RESULTS

Serological analysis

A total of 36 samples with Rose Bengal positive or negative results were selected for analysis using ELISA; the obtained ELISA results indicated that 6 samples (16.6%) had negative results. All of the tested samples had a significant IgG antibody titer against all of the tested bacterial strains, including *Proteus* ($P \leq 0.001$), *Salmonella* ($P \leq 0.001$), *Yersinia* ($P \leq 0.001$), *Pasteurella* ($P \leq 0.001$), *E. coli* ($P \leq 0.001$) and *Brucella* ($P \leq 0.001$).

The serum samples were poured in wells which had been coated with mentioned bacterial strains. After an incubation period of one hour, the anti-*Brucella* titers were assessed using an in-house ELISA. No significant ($P=0.19$) differences were observed in the assessed optical density using the absorbed ELISA against other bacteria. However, there was a significant difference ($P \leq 0.05$) between the results of the indirect and absorbed indirect ELISA for defining the *Brucella* positive serums which were evaluated on the same conditions.

The SDS-PAGE analysis showed various protein bands, ranging from 250 kDa to lower than 5 kDa in *Brucella abortus, Salmonella typhimurium*, *Pasteurella multocida*, *Yersinia enterocolitica*, *Proteus vulgaris* and *Escherichia coli*. The Western blotting of the positive serum samples revealed the obvious reactivity of the anti-*Brucella* antibodies with several protein bands in other bacteria. The western blotting assay of *Brucella* bacteria showed several immunogenic proteins although protein bands with molecular weights ranging at 63-42 kDa had more immunogenicity, followed byother protein bands with molecular weights of approximately, >17, 100 and 25 kDa, respectively. The *Proteus* protein bands with the molecular weights of respectively, >17, 25, 70, 63 and 90 kDa had more reactivity with the positive serum against *Brucella*. *Salmonella* bacteria had the immunoreactive protein bands at molecular weights of approximately, 53, >1770, 115 and >250 kDa, respectively. The *Pasteurella* protein bands with molecular weights of >17, 48, 63, 70, 75, 135, 190 and >250 kDa had more reactivity with the serum of the *Brucella*- infected sheep. Most of the immunoreactive protein bands of *Yersinia* had molecular weights of 42, >17, 58, 63 and 88 kDa, respectively. The proteins of *E. coli* with the molecular weights of >17, 30, 35, 68, 70, and 90 kDa, respectively, featured more reactivity with anti-*Brucella* antibodies. The identified common patterns in *Brucella* included immunogenic proteins, with molecular bands ranging in 63-42 kDa; as regards *Proteus,* there are two common proteins with molecular bands of 25 to 70 kDa whereas *Salmonella* manifested common 53 and 115 kDa immunoreactive proteins. Also, *Pasteurella* (135 and 190 kDA), *Yersinia* (63 and 42 kDa) and *E. coli* (30, 35 and 70 kDa) bacteria showed common immunoreactive proteins.

Bioinformatics analysis

The retrieved proteins were compared according to their amino acid sequences (AAI) and structure identity (SI). The paired sample t-test statistical analysis showed a significant difference between % sequence and the structural identity ($P \leq 0.001$). In addition, there was a significantly direct correlation between Z score and % structure identity (r= 0.74: $P \leq 0.001$) but not with the sequence identity (r=0.35; $P \leq 0.05$). Cu/Zn superoxide dismutase (SI:57.6±1.7; AAI: 56.6±1.8), methionine aminopeptidase (SI: 47.6±0.97; AAI:50.3±0.85), leucylaminopeptidase (SI: 26.1±0.29; AAI: 39.17±0.54), carbonic anhydrase (SI: 25.9±9.2; AAI: 39.4±6.04), F0F1-ATPase sub/b (SI: 24±3.20; AAI: 28.5±3.2) and cold shock domain containing protein (SI: 23.5±1.1; AAI: 30.3±0.79) showed higher structural and amino acid sequence similarity between the analyzed bacteria and *Brucella* proteins, respectively (Figure 1). Also, the amino acid sequenceand structural similarity of the two proteins, namely Cu/Zn superoxide dismutase andmethionine aminopeptidase of the mentioned bacteria, showed a significantly higher similarity to *Brucella* proteins ($P \leq 0.001$) in comparison to other analyzed proteins (Table 1).

DISCUSSION

Similar to other microbial agents, the *Brucella* infection triggers humoral immune responses against a number of antigens with shared common sequence and structure to some of other microbes and vice versa. Consequently, routine serological tests such as agglutination and ELISA may yield false positive results; in addition, it is possible to observe false negative results in the agglutination test due to prozone phenomenon. It is known as the cause of the development of the ELISA based methods using specific protein or lipopolysaccharide antigens; however, due to the genetic variability, individuals may have different humoral immune responses against the coated antigen. According to the bioinformatics analysis, there are a limited number of antigens with significant structural similarity. Detection of the IgG antibodies against *Brucella* using absorbed ELISA showed some evidence that should be taken into consideration prior to the interpretation of the serological tests. The

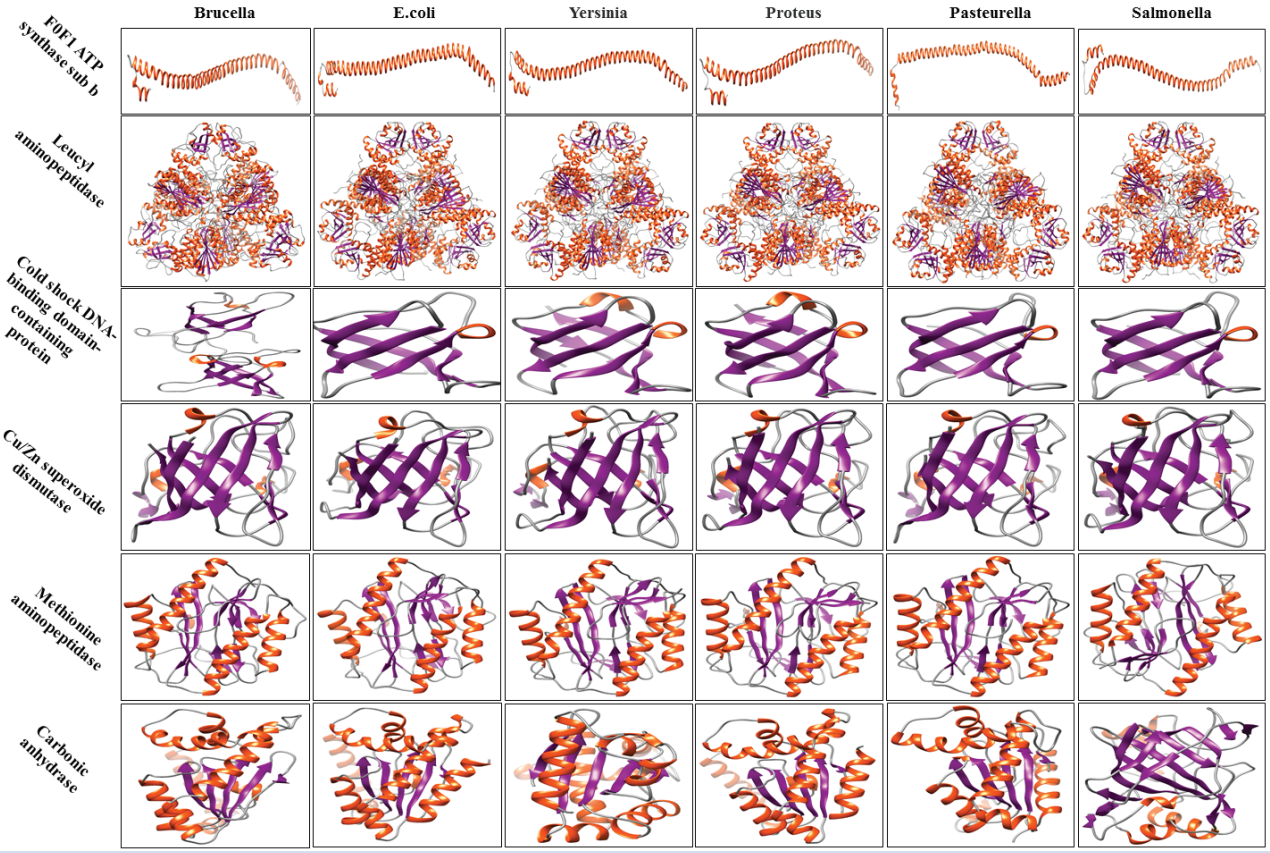

Figure 1. The three dimensional structure of five proteins with the highest similarity between the *Brucella*, *Proteus*, *Salmonella*, *Pasteurella*, *Yersinia* and *E. coli* bacteria. In images, the strand, helix and coli are shown in purple, orange and gray, respectively.

Table 1. Comparison of the amino acid sequences and structures of the immunogenic proteins of the *Brucella* with other bacteria.

Bacteria		*E.coli*	*Yersinia*	*Proteus*	*Pasteurella*	*Salmonella*
F0f1-ATPase sub/B	AAI (%)	28.21	-	37.72	23.08	25.19
	SI (%)	27.2	27.8	28.5	11	25.8
	Z score	6.7	6.7	6.7	3.683	6.7
	RMSD	1.521	1.522	1.521	1.522	1.522
solute-binding family 5 protein	AAI (%)	-	37.9	21.43	20.33	35.84
	SI (%)	9.5	35.5	12.3	8.6	34.5
	Z score	6.810	8.210	6.470	6.580	8.300
	RMSD	3.540	0.999	4.027	4.565	0.680
Outer Membrane Protein (OMP)	AAI (%)	-	-	-	-	-
	SI (%)	4.8	7.5	1.2	5.4	4.7
	Z score	2.580	3.890	2.580	2.300	0.730
	RMSD	3.815	6.475	7.797	3.129	5.324
hypothetical protein	AAI (%)	-	-	-	-	-
	SI (%)	1.4	1.0	3.7	2.9	10.6
	Z score	1.640	0.730	0.730	1.990	1.990
	RMSD	6.125	5.004	4.036	5.260	5.564
TAT_signal_seq	AAI (%)	-	-	-	-	-
	SI (%)	3.9	1.3	2.8	-	3.9
	Z score	1.640	1.640	2.580	-	1.640
	RMSD	5.010	5.487	5.375	-	4.680
DHOase	AAI (%)	23.71	24.82	24.14	-	25.00
	SI (%)	12.4	12.2	14.3	3.2	13.0
	Z score	7.240	7.240	7.240	4.250	7.240
	RMSD	2.497	2.389	2.298	10.894	2.504
serine protease	AAI (%)	-	43.82	-	37.01	-
	SI (%)	1.1	23.3	3.7	35.3	2.4
	Z score	2.300	6.700	2.580	7.240	1.990
	RMSD	5.429	4.475	5.528	2.897	6.179
short-chain dehydrogenase/ reductase	AAI (%)	28.43	28.19	32.98	24.88	25.98
	SI (%)	18.5	22.4	22.6	18.4	18.2
	Z score	7.130	7.340	7.440	7.440	7.130
	RMSD	2.969	2.746	3.116	2.863	3.179
Leucyl aminopeptidase	AAI (%)	39.30	38.57	38.19	41.18	38.95
	SI (%)	25.5	25.9	26.7	26.9	25.5
	Z score	7.740	7.740	7.740	7.740	7.740
	RMSD	2.602	2.554	2.557	2.618	2.553
cold shock domain-containing protein	AAI (%)	30.43	29.69	28.79	33.33	29.41
	SI (%)	25.0	23.6	19.2	25.0	25.0
	Z score	4.250	3.890	4.350	4.250	4.250
	RMSD	3.189	3.245	3.203	3.186	3.186
Cu/Zn superoxide dismutase	AAI (%)	51.45	57.47	55.70	55.63	62.80
	SI (%)	54.2	61.4	55.80	54.5	62.3
	Z score	6.810	7.740	7.740	7.930	7.740
	RMSD	1.214	1.263	1.260	0.176	1.134
methionine aminopeptidase	AAI (%)	52.02	49.60	48.76	48.76	52.82
	SI (%)	49.4	47.1	48.4	46.7	50.2
	Z score	7.540	7.540	7.540	7.540	7.540
	RMSD	1.182	1.249	1.179	1.214	1.174
carbonic anhydrase	AAI (%)	51.53	28.79	48.08	-	29.23%
	SI (%)	47.9	10.8	46.1	2.2	22.5%
	Z score	8.4	5.190	8.490	2.300	6.920
	RMSD	0.158	3.515	0.155	5.881	2.606

(-) No reliable sequence or notable similarity has been found.

anti-*Brucella* antibodies titer elevated significantly after removing the cross-reacting antibodies against the tested bacteria; however, there were variations in the tested serums. These observations may be due to the presence of the cross-reacting IgM antibodies, different antibodies titers against other bacteria in each sample or different immunogenicity of the cross reactive antigens of the *Brucella* among the tested animals. The analyzed bacteria in the western blot analysis showed a few common bands in positive serum samples such as >17, 25, 35, 63, 70 and 100 kDa protein bands. Hence, it is suggested that animals have common and but also, individual cross-reacting antibodies against the tested bacteria. Consequently, the current serological tests such as agglutination test or whole cell ELISA could not differentiate the cross-reacting antigens. In addition, the results of the current study introduced some antigens with 100% immunoreactivity among sheep via the western blot analysis which could create a specific pattern among the analyzed bacteria. The previous investigations had revealed five cytosoluble proteins bands of 19, 24, 28, 32, and 54 kDa of *B. melitensis* B115 (rough) that could differentiate the vaccinated, naturally and experimentally infected sheep with different *B. melitensis* variants. These researchers reported the 28-kDa antigen as the earliest and the most consistent humoral responses (100%) during a natural infection. The *Brucella*-free ewes had antibody against the protein bands of 39 and 50 kDa and the presence of the nonspecific epitopes on these proteins was unveiled in immunoblot and ELISA (Salih-AljDebbarh et al., 1996).

According to the absorb ELISA results, the majority of the cross-reacting antibodies belong to the IgM class which are often produced against the outer LPS membrane of bacteria; furthermore, the IgM targeted antigens are the cross-reactive antigens that manifest reactive IgG antibodies in positive samples in addition to IgM. The humoral immune response against outer membrane proteins in *B. melitensis*is reported as low and heterogeneous (Zygmunt et al., 1994). Previously, Falcaoet al. (2019) noted the cross reaction in vaccinated and *Brucella* infected animals; other researchers have also reported the cross reacting antibodies due to the LPS-O antigen present in the B19 vaccine and gram-negative bacteria (Nielsen et al., 2004; Al Dahouk et al., 2006; Ko et al., 2012). The development of the diagnostic test without LPS-O antigens could reduce the costs associated with vaccination, diagnosis and slaughter of positive animals (Pajuaba et al., 2012). Also, different non-LPS antigens belonging to the outer membrane, such as BP26/omp28, are regarded as immunodominant common antigens in infected cattle, goat, dogs and human (Rossetti et al., 1996; Chaudhuri et al., 2010). Another research has regarded the *Brucella* OMP25/OMP31 family antigens as excellent immunogenic antigens and suggested these proteins as targets in diagnostic kits and protective vaccines (Gomez et al., 2013). Moreover, another study has reported the immunoreactivity of the goat, sheep and rabbit sera against proteins with molecular weights less than 20, 32-34 and 45 KDa; these proteins are the most abundant proteins of S19 strain and can be considered as diagnostic antigens or vaccines (Mostafaie et al., 2005). In addition, previously, proteins with a molecular weight of 14 KDa anda 18-20 KDa protein of *B. abortus* induced humoral immune responses and protected mice against virulent *B. abortus* (Chirhart-Gillelandet al., 1998). Seven outer membrane proteins with molecular weights of 10, 16, 19, 25 to 27, 31 to 34, 36 to 38, and 89 kDa were reported in the rough and smooth strains of *B. abortus*; except 31 to 34 and 89 kDa, other proteins in smooth strains could react with IgG. OMP28 is another conserved outer membrane proteins of *Brucella* that is considered as an important diagnostic antigen (Manat et al., 2016).

An antigen, which is present in the cytosol of the *Brucella* species is a 24-kDa protein (CP24), is capable of differentiating the vaccinated from the infected sheep (Cassataro et al., 2002); however, it is a homologue of *E. coli* ribosome recycling factor (RRF) and other species. Another study reported it as a cross-reacting antigen among the infected and *Brucella*-free patients (Delpino et al., 2003). The utilization of different species, samples and bacterial strainsis the main reason for the diversity of the attained results. A protein named BP2 was successfully utilized for the detection of the sheep infected with *B. melitensis*or *B. ovis* (Lindler et al., 1996). Common antigens which are shared among all *Brucella* species, such as lumazine synthase (BLS) and 18-kDa cytoplasmic protein were used for the preparation of the diagnostic test of human and canine brucellosis (Goldbaum et al., 1999). Using the western blot analysis, some proteins with a molecular weight lower than 20 kDa were identified as the specific markers of the animals infected with *B. abortus* (Pajuaba et al., 2012; Kim et al., 2014). Due to the diverse immunogenicity of most of the proteins in individuals, the employment of more than one specific and immunogenic protein

may reduce the incidence of false results in serological analyses; our analysis revealed common bacterial protein bands with different immunogenicity in *Brucella* positive serum samples.

According to OIE (2021a), sensitivity and specificity values greater than 95% could be accepted as a diagnostic assay. Based on this principle, WB was compared with other serological tests. In comparison to the Rose Bengal test, Pajuaba et al. (2012) reported 99% specificity and 93% sensitivity for WB analysis. A research suggested the production of the reactive antibody against small and large sized polypeptides in the acute and chronic phases of the *Brucella* infection, respectively unlike the 73, 66, 46, 43 and 33 kDa polypeptides that were detected in both forms of the brucellosis (Alaidan et al., 2019). These authors interestingly showed the persistence of the reactive antibody against 43 and 66 kDa polypeptides after therapy, with the induction of the memory immune cells being considered as the chief cause. Polypeptides with similar molecular weights also featured immunoreactivity in the current study. In consistent with our results, the previous immunoblot analyses had discovered variations in human and animal immune responses against the *Brucella* proteins (Debbarh et al., 1995; Schumaker et al., 2010) although animal genetics, infectious dose, and the infectious conditions of the endemic area were reported as the effective factors in these variations.

The bioinformatics analysis revealed six immunogenic proteins (KO et al., 2012) with the highest sequence and structural similarities with *Proteus*, *Salmonella*, *Pasteurella*, *Yersinia* and *E. coli*. Even though Ko et al. (2012) considered these proteins as specific and immunogenic antigens, the three dimensional alignment showed asignificant similarity in the structures of proteins: thus, Cu/Zn superoxide dismutase and methionine aminopeptidase proteins could be the targets for multi organism bacterial immunization. The western blotting analysis showed cross reactive protein bands with similar molecular weights as the proposed proteins for bioinformatics analyses; however, due to the lack of access to protein sequencing, this issue was not cited with certainty. The hypothetical protein with the lowest Z score could be mentioned as a specific antigen for the diagnostic tests of brucellosis.

CONCLUSION

The current research analyzed the cross reactivity of the Rose Bengal positive serum samples to other bacteria. Altogether, ELISA, absorbed ELISA, and the Western blot assay unveiled a common ground to be utilized for assessment. The cross reactive antibodies belong to both IgM and IgG isotypes. The bioinformatics analysis identified the six *Brucella* proteins with the highest sequence and structure similarity with other bacteria, including Cu/Zn superoxide dismutase, methionine aminopeptidase, leucylaminopeptidase, carbonic anhydrase, F0F1-ATPase sub/b and cold shock domain containing protein.

CONFLICT OF INTEREST

The authors of this manuscript declare no conflict of interests.

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