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PRODUCTION OF HYPERIMMUNE SERUM AGAINST RECOMBINANT PROTEINS SODC AND OMP16 OF BRUCELLA SPP. FOR THE DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TEST SYSTEM

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ABSTRACT

Brucellosis is a widespread zoonotic disease transmitted from domestic animals to humans and is endemic in many regions of the world. There is a need for highly specific serological tests for brucellosis based on recombinant proteins to reduce cross-reactivity with other Gram-negative bacteria and to differentiate between vaccinated and infected individuals. The aim of the present study was to obtain hyperimmune sera against *Brucella* spp. antigens in laboratory animals. Rabbits and guinea pigs were used as experimental animals. For immunization, recombinant *Brucella* spp. proteins Omp16 and SodC were used in combination with adjuvants, including aluminum hydroxide ($\text{Al}(\text{OH})_3$), as well as complete and incomplete Freund's adjuvant (CFA/IFA). Immunization was performed according to a standard protocol, followed by monitoring of antibody levels in blood sera using enzyme-linked immunosorbent assay (ELISA) and agar gel precipitation test (AGPT). For protein expression, producer strains were cultured in LB medium supplemented with kanamycin. Protein synthesis was induced with 0.1 mM IPTG. Purification was performed using immobilized metal affinity chromatography (IMAC).

As a result of the study, hyperimmune sera with a high level of specific antibodies were obtained. It was demonstrated that the use of adjuvants promotes the development of a pronounced humoral immune response in immunized animals. The highest antibody titers were observed in animals immunized with CFA/IFA adjuvants, where antibody titers reached 1:6400 in rabbits and 1:1600 in guinea pigs. When aluminum hydroxide was used, antibody titers were 1:3200 and 1:800 in rabbits and guinea pigs, respectively.

The obtained results confirm the effectiveness of the proposed approach for producing hyperimmune sera and demonstrate their potential for use as reference positive controls in the development and standardization of serological methods for brucellosis diagnostics.

Keywords: recombinant proteins SodC and Omp16, immunogenicity, hyperimmune serum, enzyme-linked immunosorbent assay (ELISA), immunization.

INTRODUCTION

Brucellosis is classified as a particularly dangerous bacterial zoonosis widely distributed among farm animals and humans. The infection is transmitted through contact with animals or the consumption of contaminated animal products, which determines its medical and veterinary significance [1-2].

Currently, twelve species have been described. These include *Brucella abortus* (cattle), *B. melitensis* (sheep/goats), *B. suis* (pigs), *B. canis* (dogs), *B. ovis* (rams), and *B. neotomae* (rats). Recently, six additional species have been added to the genus, including *B. ceti*, *B. pinnipedialis*, *B. inopinata*, *B. microti*, *B. paponis*, and *B. vulpis* [3].

According to the World Health Organization (WHO), more than 500,000 new cases of brucellosis are reported worldwide each year, while the actual incidence in endemic regions may be several times higher than official statistics [4].

Brucellosis remains one of the most significant zoonotic infections, characterized by a chronic course and substantial socio-economic losses in livestock production and public health sectors [5]. In recent years, approaches to the development of diagnostic test systems based on recombinant antigens with high specificity and reproducibility have been actively advancing. Recombinant *Brucella* spp. proteins are widely considered promising components of vaccines and diagnostic platforms due to their conservation and strong an-

tigenic properties [6]. Among the most studied immunodominant proteins are the ribosomal protein L7/L12, Cu/Zn superoxide dismutase (SOD), as well as outer membrane proteins Omp16 and Omp19, which demonstrate strong immunogenicity and diagnostic relevance [7].

Outer membrane protein Omp16 is an essential component of the *Brucella* cell envelope and plays a crucial role in the pathogenesis of brucellosis [8]. Omp16 functions as a pathogen-associated molecular pattern (PAMP), activates innate immune cells, and subsequently induces antigen-specific adaptive immune responses characterized by T-cell activation and the production of specific antibodies [9]. Owing to these properties, Omp16 is considered a promising recombinant antigen for the serological diagnosis of brucellosis.

The potential of the recombinant SodC protein for the serological diagnosis of brucellosis has been demonstrated in studies showing its high specificity for antibody detection by ELISA and the comparability of its diagnostic performance with that of the serum agglutination test (SAT). [10].

Effective control of brucellosis depends on timely and accurate diagnosis. Currently, several diagnostic methods are used for brucellosis detection, including serological assays such as the Rose Bengal Test (RBT), the Serum Agglutination Test (SAT), enzyme-linked immunosorbent assay (ELISA), and molecular methods such as polymerase chain reaction (PCR) [11]. For serological testing, specific hyperimmune

serum against the corresponding disease is required.

Further studies using vector-based vaccines have shown that the expression of L7/L12, Omp16, Omp19, and SodC proteins induces antigen-specific humoral and cellular immune responses and provides protection in experimental animals against *Brucella* infection, confirming their high immunogenicity and potential for diagnostic development [12].

Taken together, these data indicate that the listed proteins are promising candidates for the development of ELISA test systems aimed at evaluating post-vaccination immunogenicity in preclinical studies.

Further evidence supporting the diagnostic potential of Omp16 was provided by the study of Golchin et al. (2022), who developed an indirect ELISA based on the recombinant Omp16 protein. When compared with a commercially available ELISA kit for the detection of anti-*Brucella* IgG antibodies (Pishtazteb, Tehran, Iran), which uses *Brucella* lipopolysaccharide (LPS) as the coating antigen, the developed assay demonstrated 100% sensitivity and 100% specificity [13].

Despite the accumulated knowledge on the antigenic properties of individual recombinant *Brucella* spp. proteins, data on their comparative immunogenicity and ability to induce a high-titer humoral response remain limited and fragmented. In particular, there is evidence regarding the immunoreactivity and potential diagnostic significance of SodC and Omp16 proteins; however, studies addressing their comprehensive evaluation of immunogenicity under conditions of hyperimmune serum production and subsequent application in serological test systems remain limited [14].

Accordingly, the aim of our study is to obtain hyperimmune serum against recombinant SodC and Omp16 proteins of *Brucella* spp. and to evaluate its suitability for the development of an enzyme-linked immunosorbent assay (ELISA) system intended for the assessment of post-vaccination immune responses.

MATERIALS AND METHODS

Expression and purification of recombinant *Brucella* spp. proteins

Expression of recombinant proteins in the producer *Brucella* spp. strain was carried out in LB medium at 37 °C with shaking at 250 rpm until an OD₆₀₀ of approximately 0.8 was reached. Induction was performed by adding IPTG to a final concentration of 0.1 mM, followed by incubation for 2 h. Cells were harvested by centrifugation and subjected to lysis in lysis buffer containing lysozyme, followed by centrifugation to remove cell debris. Recombinant proteins were purified by immobilized metal affinity chromatography (IMAC) on Ni²⁺-agarose with elution using imidazole. Further purification and desalting were performed by gel filtration on Sephadex G-25. The purity of the fractions was assessed by SDS-PAGE. The preparation was sterilized by filtration through 0.22 µm membrane filters and stored at -70 °C [15].

Expression of the target protein was monitored by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) [16]. Specificity was confirmed by Western blot analysis using specific antibodies against the target protein [17]. Protein concentration was determined by the Lowry method (Lowry et al., 1951) [18].

Animals. Clinically healthy guinea pigs weighing 300–400 g and Chinchilla rabbits weighing 2.0–2.5 kg were used for the production of hyperimmune serum against recombinant *Brucella* spp. proteins. A total of 18 animals of each species were used. The animals were randomly divided into two experimental groups according to different immunization schemes and two control groups, with three animals per group.

The animals were housed under standard vivarium conditions in compliance with biosafety requirements and the principles of humane treatment of laboratory animals.

Immunization protocol. Immunization was carried out using recombinant *Brucella* spp. proteins that had been previously purified and dialyzed.

Primary immunization of the animals was performed with antigen at a concentration of 50 µg/mL without the use of an adjuvant. The preparation was administered at three sites: into the popliteal lymph node region of the hind limbs, the coccygeal region, and subcutaneously on the back. The injection volume was 0.5 mL for rabbits and 0.3 mL for guinea pigs.

Hyperimmunization was performed 14 days after primary immunization using increasing doses of antigen in combination with adjuvants. Aluminum hydroxide was used for the first experimental group, while complete and incomplete Freund's adjuvant were used for the second group. Booster injections were administered three times at 7-day intervals (on days 14, 21, and 28).

Seven days after each immunization, blood samples were collected from the jugular vein of the animals.

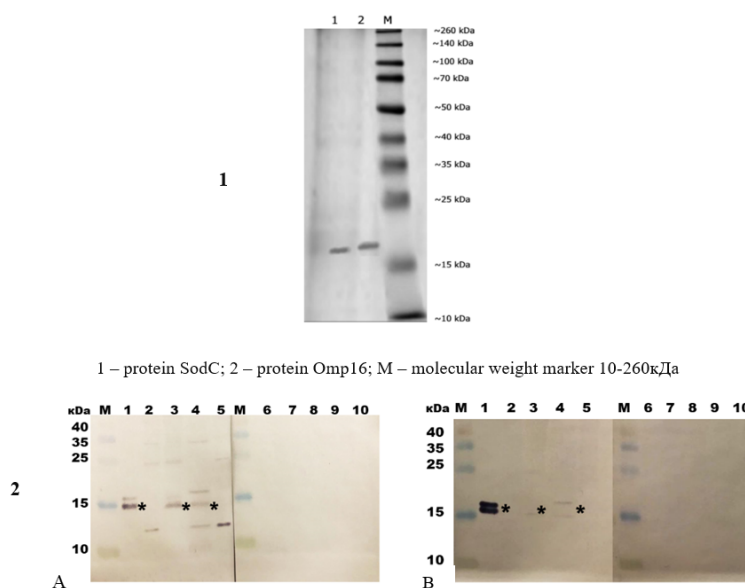
The specific activity of the obtained hyperimmune serum was determined by enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) using the corresponding recombinant antigen.

All animal procedures were conducted in accordance with national and international guidelines for the care and use of laboratory animals. The study protocol was approved by the local Bioethics Committee of the Research Institute for Biological Safety Problems.

Assessment of immunogenicity

For ELISA, 96-well strip microplates (Nunc PolySorp) were used. The plates were coated with recombinant proteins at a concentration of 1 µg/mL in carbonate buffer and incubated overnight at +4 °C (16–18 h). After incubation, the wells were washed three times by adding 300 µL of washing buffer to each well and blocked with 1% bovine serum albumin (BSA) solution for 1 h at 37 °C. After washing, the wells were washed three times. Test serum samples were added in serial dilutions and incubated for 1 h at 37 °C. After five washes with washing buffer, an HRP-conjugated reagent for IgG detection was added to the wells and incubated for 1 h at 37 °C. The plates were then washed again five times, and color development was performed using the substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). After 20 ± 5 min, the reaction was stopped by adding stop solution. Optical density was measured using an automated microplate reader at a wavelength of 405 nm.

The agar gel immunodiffusion (AGID) test was performed on 1.5% Difco agar prepared in 0.15 M sodium chloride solution. Wells were cut in the agar using a “star-shaped” template



1 – protein SodC; 2 – protein Omp16; M – molecular weight marker 10-260 kDa
 A – protein Omp16; B – protein SodC; M – protein molecular weight marker. 1, 6 – positive control; 2, 7 – *E. coli* cell lysate before IPTG induction; 3, 8 – *E. coli* cell lysate 2 h after IPTG induction; 4, 9 – *E. coli* cell lysate 4 h after IPTG induction; 5, 10 – lysate of non-transformed *E. coli* cells; 1-5 – specific serum against the recombinant protein; 6-10 – normal serum.

Figure 1 – Analysis of purified recombinant Omp16 and SodC proteins of *Brucella* spp.

1 – SDS-PAGE; 2 – Western blot.

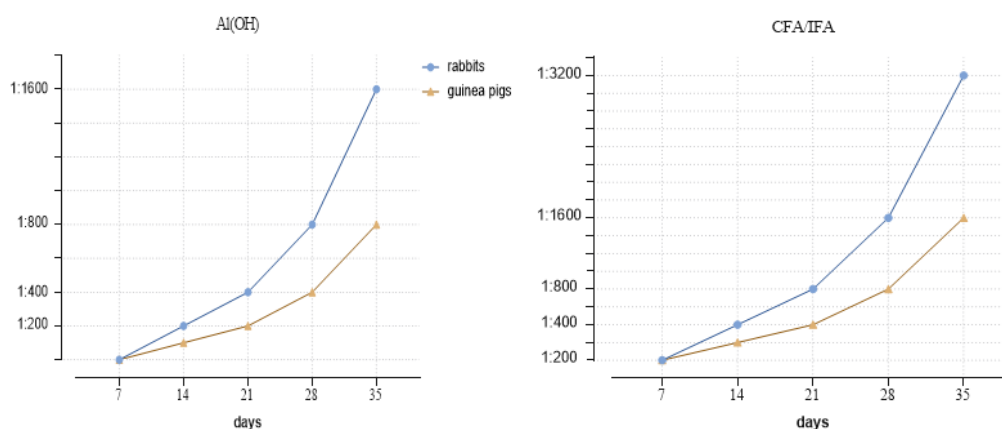


Figure 2. Antibody titer of hyperimmune sera against recombinant SodC protein in ELISA.

containing one central and six peripheral wells. The excised agar plugs were removed using a needle.

The peripheral wells of the “star-shaped” pattern were filled with test samples in serial dilutions up to 1:32. The central well was filled with proteins at a concentration of 50 µg/mL.

After setting up the reaction, the Petri dishes were covered with lids, placed under a glass bell jar with a moistened cotton swab (humid chamber), and incubated at room temperature (22 ± 2 °C) for 24–48 h.

The results of the agar gel immunodiffusion (AGID) test were analyzed qualitatively based on the presence or absence of a precipitation line between the antigen and the test serum.

Statistical analysis

Processing and analysis of experimental data were performed using Microsoft Excel and GraphPad Prism version 8.0 [19]. Group comparisons were performed using Student's t-test [20]. Differences were considered statistically significant

at $p < 0.05$. The correlation between ELISA results and AGID outcomes was assessed using Pearson's correlation coefficient.

RESULTS

Expression and purification of recombinant proteins

Purification was performed on Ni-NTA agarose using His-Trap columns, based on the binding interaction between nickel ions and histidine residues. Induction of target gene expression resulted in the synthesis of SodC and Omp16 proteins. The molecular weights of the recombinant proteins were 18 kDa and 19 kDa, respectively, as determined by SDS-PAGE (Figure 1). Western blot analysis confirmed the specificity of the obtained proteins. The concentration of the purified proteins was 280 µg/mL.

Antibody production in immunized animals

As a result of immunization of laboratory animals with recombinant *Brucella* spp. proteins, hyperimmune sera with pronounced specific activity were obtained. The antigen dose

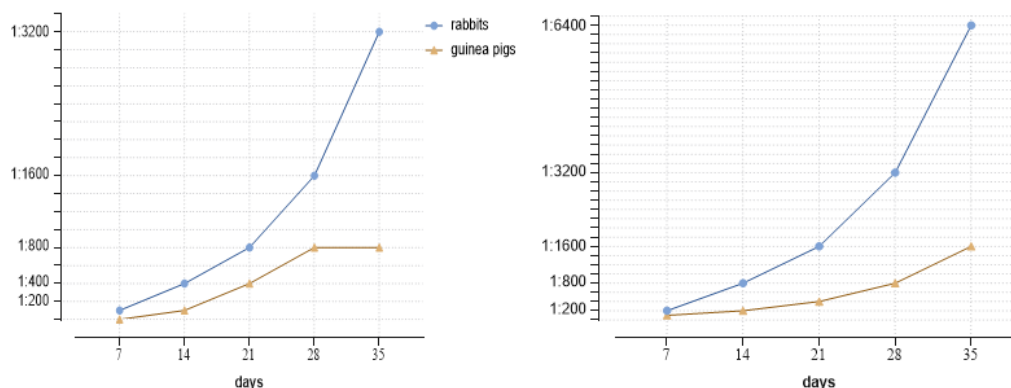


Figure 3. Antibody titer of hyperimmune sera against recombinant Omp16 protein in ELISA.

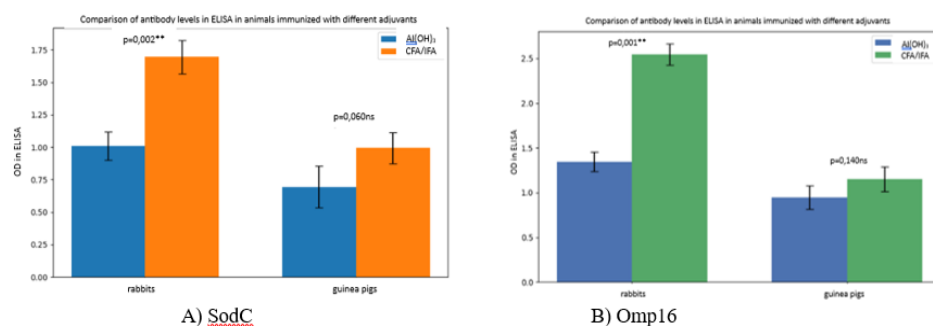


Figure 4. Statistical analysis of ELISA data using Student's t-test.

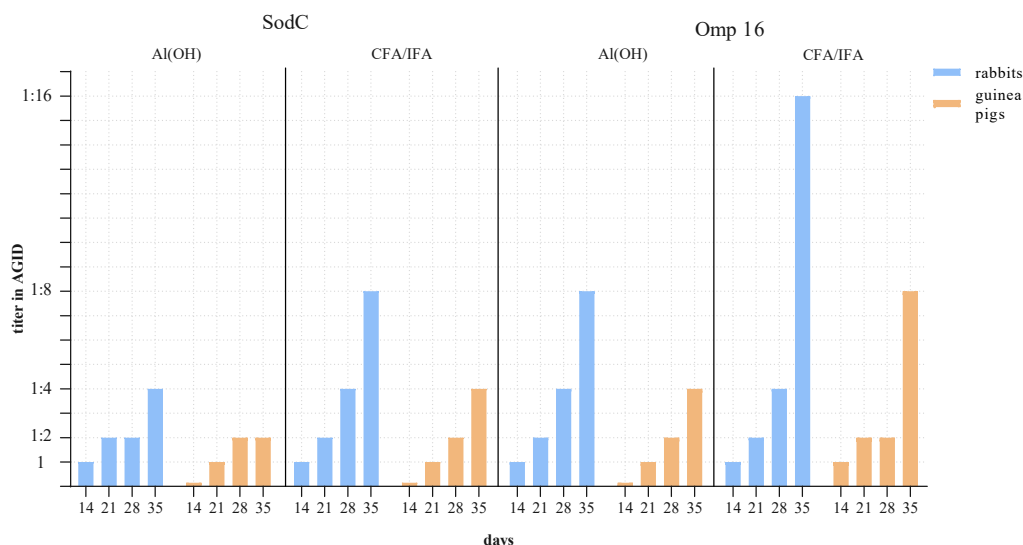


Figure 5. Antibody titers of hyperimmune sera in the AGID test.

used for hyperimmunization was 100–200 µg for rabbits and 70–100 µg for guinea pigs. Aluminum hydroxide was used at a final concentration of 0.2%, while CFA and IFA were applied in a 1:1 ratio.

Based on the ELISA results, a gradual increase in the level of specific antibodies was observed after primary immunization, which significantly increased following booster injections. The highest optical density (OD405) values were recorded 7 days after the third booster injection (Figure 2 and 3).

Statistical analysis of the ELISA data is presented in

Figure 4. Data are presented as mean ± standard deviation (**Mean ± SD**). In the experiment with the SodC protein (Figure 4A), rabbits immunized with CFA/IFA showed a significantly higher antibody level compared to the Al(OH)₃ group ($p = 0.002$), whereas in guinea pigs the differences between the groups were not statistically significant ($p = 0.060$). In the Omp16 experiment (Figure 4B), differences between groups in rabbits were highly significant ($p < 0.001$), whereas in guinea pigs the differences did not reach statistical significance ($p = 0.14$).

The antibody titers determined by the AGID test are

presented in Figure 5. The results of the AGID test demonstrated the formation of a distinct precipitation line between the hyperimmune serum and the corresponding recombinant antigen, confirming the specificity of the obtained antibodies. Negative control sera did not produce any precipitation lines.

The correlation between ELISA results and AGID test was assessed using Pearson's correlation coefficient. A strong positive correlation was observed between antibody titers ($r = 1.0$), indicating high agreement between the methods.

The results of the experiment showed that the use of the CFA/IFA adjuvant system resulted in a more pronounced antibody production compared to $Al(OH)_3$. In addition, the antibody response in rabbits was significantly higher compared to guinea pigs when both adjuvants were used.

As a result of the study, an active hyperimmune serum against recombinant *Brucella* spp. proteins was obtained in rabbits. Immunization with the Omp16 protein in combination with the CFA/IFA adjuvant system induced a pronounced humoral immune response, with antibody titers in ELISA reaching 1:6400 in rabbits and 1:1600 in guinea pigs. Immunization with recombinant SodC protein using the CFA/IFA adjuvant system also induced a specific immune response, with antibody titers of 1:3200 and 1:800.

Thus, the conducted studies demonstrated that immunization of animals with SodC and Omp16 antigens of *Brucella* spp. in combination with adjuvants induces a pronounced humoral immune response and enables the production of highly active hyperimmune sera suitable for use in serological diagnostic methods.

DISCUSSION

Currently, research on brucellosis is actively focused on the identification and characterization of non-lipopolysaccharide (LPS-independent) antigens that can be used both for specific serological diagnosis of the disease and for the development of novel vaccine candidates. Traditional serological tests based on the lipopolysaccharide antigen of *Brucella* have high sensitivity; however, they are often characterized by cross-reactions with other Gram-negative bacteria, which reduces their diagnostic specificity. In this regard, protein antigens are of particular interest, as their use can reduce the likelihood of cross-reactivity and improve diagnostic accuracy. Among them, outer membrane proteins (Omps) are considered the most promising, as they are major immunoreactive components of the bacterial cell and are actively investigated as candidates for the development of diagnostic test systems and vaccine preparations.

The need to develop highly specific serological tests for brucellosis based on *Brucella* antigens has been emphasized by a number of researchers. In particular, J.P. Gorvel notes that the use of outer membrane proteins, as well as inner membrane, cytoplasmic, and ribosomal proteins, may help reduce cross-reactivity with other Gram-negative microorganisms and allow differentiation between post-vaccination immunity and infection [21].

In the study by J.J. Letesson, eight **Brucella** proteins (Omp36, Omp25, Omp19, Omp16, Omp10, p17, p15, and p39) were investigated as potential antigens for serological diagnosis. The obtained results demonstrated that non-lipo-

polysaccharide protein antigens may be considered a promising alternative to traditional LPS antigens. Among the studied recombinant proteins, p17, p15, and p39 showed the highest diagnostic informativeness, confirming the potential use of recombinant proteins as immunogens for the production of hyperimmune sera and the development of new diagnostic test systems [22].

Similar results were obtained in the study by Sung et al. (2014), where recombinant SodC and Omp2b proteins of *Brucella abortus* were used as antigens for the serological diagnosis of brucellosis. Immunization of mice with these antigens in combination with complete and incomplete Freund's adjuvant induced a strong cellular and humoral immune response. At the same time, a higher level of antibodies was observed with the use of the recombinant outer membrane protein rOmp2b compared to Cu/Zn superoxide dismutase (SodC). The obtained data indicate the high immunogenicity of the studied proteins and confirm their potential as LPS-independent antigens for the development of new serological tests [23].

The results obtained in the present study are consistent with those of the aforementioned works. The experiment demonstrated that immunization of animals with recombinant *Brucella* proteins also induces a pronounced humoral immune response accompanied by the accumulation of specific antibodies. This confirms the high immunogenicity of the studied proteins and their potential as antigens for the production of hyperimmune sera. Thus, the use of recombinant *Brucella* proteins can be considered a promising approach for the development of more specific diagnostic test systems and the improvement of serological diagnosis of brucellosis.

CONCLUSION

Due to their high immunogenicity, conserved nature, and ability to induce robust immune responses, the recombinant proteins Omp16 and SodC are considered promising antigens for the development of highly sensitive and highly specific serological assays for the diagnosis of brucellosis in animals and humans.

The obtained results are consistent with previous studies demonstrating that the recombinant Omp16 protein exhibits high antigenicity and immunogenicity and contributes to the development of protective immune responses, making it a promising component of serological diagnostic assays and subunit vaccines [24]. The SodC protein is also recognized as an immunodominant antigen of *Brucella* spp., induces the production of specific antibodies in infected animals, and is considered a promising diagnostic marker as well as a potential component of subunit vaccines [25]. The results of the present study demonstrate the high immunogenicity of the recombinant proteins investigated and the effectiveness of the adjuvant systems used to induce specific antibody production. The resulting hyperimmune sera exhibited high antigen-specific reactivity and can serve as reference positive sera for the development, optimization, and validation of enzyme-linked immunosorbent assay (ELISA)-based diagnostic systems for brucellosis.

Thus, the use of recombinant Omp16 and SodC proteins as immunogens represents a promising approach for the production of standard diagnostic reagents and the further im-

provement of laboratory diagnostic methods for brucellosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.Zh.K.; methodology, O.N.K., I.A.U., B.A.K., U.B.K., and I.A.A.; statistical analysis, O.N.K., S.O.N., K.M.Zh., and U.B.K.; study design, O.N.K., S.O.N. and I.A.A.; data interpretation, K.M.Zh., A.A.K., B.A.K., and I.A.U.; writing-original draft preparation, O.N.K., U.B.K., and I.A.A.; writing-review and editing, K.Zh.K. and O.N.K.; supervision, Ch.O.V.

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All authors have read and agreed to the published version of the manuscript.

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