Full Length Research Article

Production of Recombinant Antigens for Use in the Serological Diagnosis of Animal Brucellosis

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ABSTRACT

Background: Brucellosis, a global zoonotic disease, poses significant threats to both public health and economic stability, particularly in livestock industries, necessitating the development of improved diagnostic methods for early detection and control. The development of recombinant antigens offers a promising approach to improve diagnostic accuracy and standardization of test systems.

Methods: Genes encoding *Brucella* antigens (Omp2b, BP26, and SodC) were cloned into the pET-28a(+) vector and expressed in Escherichia coli BL21(DE3). Recombinant proteins were purified using Ni- NTA chromatography and characterized for expression level, solubility, yield, and stability. Diagnostic performance was evaluated using the same complete bovine serum panel of 86 samples, comprising 56 serologically positive and 30 serologically negative sera. Each of the 86 serum samples was tested with all three individual antigens (Omp2b, BP26, and SodC) and all three combined-antigen formulations (BP26+Omp2b, BP26+SodC, and BP26+Omp2b+SodC); no separate sub-panels were used. Sensitivity and specificity were therefore calculated for every antigen format using the same reference panel, followed by comparisons with commercial ELISA kits. Paired differences in sensitivity and specificity were evaluated using Cochran’s Q test followed by Holm-adjusted pairwise McNemar tests.

Results: Successful cloning and expression yielded proteins of expected molecular weights (38, 26, and 18 kDa). BP26 demonstrated the highest expression (28.4%), solubility (82.1%), and yield (14.8 mg/ L), along with superior stability and reproducibility. The verified sensitivity/specificity values were 80.6%/88% for Omp2b, 91.7%/95% for BP26, 75%/90% for SodC, 94.4%/92% for BP26+Omp2b, 95.8%/93% for BP26+SodC, and 97.2%/91% for BP26+Omp2b+SodC. The new tests also showed improved stability, reduced cross- reactivity, and better detection at low antibody titre compared to commercial kits.

Conclusion: Recombinant antigens, particularly BP26 and its combinations, show improved diagnostic performance in the serological diagnosis of animal brucellosis. The developed antigen systems demonstrate high sensitivity, specificity, and stability, supporting their potential application in advanced diagnostic test development.

INTRODUCTION

The relevance of this study is driven by the need to improve the diagnosis of animal brucellosis, which remains one of the most significant zoonotic infections worldwide. The disease causes substantial economic losses in livestock production due to reduced productivity, reproductive disorders, and the compulsory culling of affected animals. In Kazakhstan and other countries with well- developed agricultural sectors, the problem is particularly acute, as the epidemiological situation is complicated by the widespread distribution of the pathogen and the difficulties associated with the timely detection of infected animals. Traditional serological methods, such as agglutination tests and enzyme- linked immunoassays, have limitations in terms of sensitivity and specificity, which hinder the accurate identification of early and latent stages of infection. The issue addressed in this study is linked to the insufficient effectiveness of existing commercial tests, which do not always reliably distinguish positive from negative samples and often produce false-positive or false-negative results. The situation is especially critical when antibody titre are low, where the sensitivity of standard kits is insufficient for the detection of infected animals. In addition, the antigenic preparations currently in use are characterized by heterogeneity, production complexity, and instability during storage, all of which reduce their practical value. The absence of highly sensitive and standardized diagnostic tools limits the implementation of effective brucellosis control and eradication programs.

In the study by Ingirbai et al. [1], it was shown that *Brucella* multiprotein antigens exhibit high immunoreactivity when interacting with positive sera. These findings supported the feasibility of using recombinant proteins as the basis for improved diagnostic systems. Building on the results of Mukantayev et al. [2], it was demonstrated that the recombinant Rap1 protein, applied in a lateral- flow immunoassay format, provided rapid and accurate detection of bovine babesiosis in Kazakhstan. These data confirmed the rationale for developing similar rapid tests based on recombinant antigens to enhance the efficiency of serological diagnostics for other infectious diseases.

In the study by Bulashev et al. [3], a validation of an enzyme- linked immunoassay based on chimeric antigens was carried out, confirming the high reproducibility of the method under conditions in Kazakhstan. The findings demonstrated the practical applicability of such tests in national monitoring programs. When considering the data presented by Souza et al. [4], it was established that a rapid test based on the recombinant *Brucella* protein 26 (BP26) ensured high analytical performance during field trials. This demonstrated that the use of individual antigens may also be a promising approach for the development of rapid diagnostic systems.

According to the review by Elrashedy et al. [5], attention was focused on modern approaches to the control and diagnosis of brucellosis, including the use of recombinant proteins and new vaccine strategies. The analysis emphasized the need to shift from traditional serological methods to more standardized and specific technologies. Based on the research of Bányász et al. [6], it was established that the Ghostbuster method, which is based on a Western blot panel, effectively eliminates the problem of false- positive results in the serological diagnosis of brucellosis. This highlighted the importance of improving approaches to the confirmation of serological tests in order to enhance diagnostic accuracy. Building on the results of Bedir et al. [7], a new lateral- flow immunochromatographic system was developed and evaluated for the diagnosis of brucellosis in different animal species. The test was shown to be highly versatile and reliable under field conditions. Data presented by Andrade et al. [8] demonstrated that the use of recombinant enzymatic proteins – malate dehydrogenase and superoxide dismutase – in an indirect enzyme-linked immunoassay format provided a high level of informativeness. This indicated the potential for using such enzymes as alternative diagnostic targets.

In the study by Fadaie et al. [9], the immunogenicity of a multi-epitope phage vaccine against *Brucella* spp. was evaluated in a mouse model. It was demonstrated that this strategy induces a pronounced *in vivo* immune response and may serve as a basis for further improvement of diagnostic and preventive approaches. Based on the findings of Novak et al. [10], a new glycoprotein immunochromatographic test was developed, providing rapid and reliable results. This approach opened up prospects for the use of glycoproteins in rapid diagnostics.

According to the analysis by Bányász et al. [11], false-positive results in serological tests for brucellosis may arise due to the use of unsuitable antigenic determinants, which significantly reduce diagnostic reliability. This conclusion highlighted the need for more careful antigen selection in order to minimize errors in serological detection. In the review by Qureshi et al. [12],

information on the epidemiology, pathogenesis, diagnosis, and treatment of brucellosis was systematized. The study emphasized the importance of combining serological and molecular methods to improve infection control. Insufficiently explored issues include the long- term stability of recombinant antigens during storage, the optimization of antigen combinations for universal serological tests, and the application of the developed systems in large- scale field monitoring. This research aimed to evaluate *Brucella* recombinant antigens for the development of more accurate serological tests. The objectives included cloning and expression of *Brucella* antigen genes, production and characterization of recombinant proteins, and comparison of the diagnostic performance of the developed tests with commercial kits.

METHODS

Antigen selection and cloning

For the purposes of this study, serum status was determined using a composite reference standard rather than the result of a single serological assay. A sample was classified as reference-positive when it produced a positive Rose Bengal plate test result that was confirmed by at least one additional standard serological method, namely the serum agglutination test, complement fixation test, or commercial ELISA; positive classification also required epidemiological confirmation of brucellosis at the herd level. A sample was classified as reference-negative when it originated from a farm officially recognized as free of brucellosis and had negative results in the Rose Bengal plate test and the confirmatory serological tests used in the routine diagnostic scheme. Archived samples were included only when the same diagnostic and epidemiological criteria were documented. This predefined composite classification constituted the reference standard for calculating sensitivity and specificity. The same complete panel was used for the assessment of every individual antigen and antigen combination. Thus, each serum sample was analyzed with Omp2b, BP26, SodC, BP26+Omp2b, BP26+SodC, and BP26+Omp2b+SodC, and no antigen-specific sub-panels were formed. For the purposes of this study, serum status was determined using a composite reference standard rather than the result of a single serological assay. A sample was classified as reference-positive when it produced a positive Rose Bengal plate test result and this result was confirmed by at least one additional standard serological method, namely the serum agglutination test, complement fixation test, or commercial ELISA. Positive classification also required epidemiological confirmation of brucellosis at the herd level. A sample was classified as reference-negative when it originated from a farm officially recognized as free of brucellosis and had previously produced negative results in the Rose Bengal plate test and the confirmatory serological tests used in the routine diagnostic scheme. Archived samples were included only when the same diagnostic and epidemiological criteria were documented. This predefined composite classification constituted the reference standard against which the sensitivity and specificity of the recombinant-antigen assays were calculated. Molecular cloning was performed using the restriction enzymes NcoI and XhoI, T4 DNA ligase, and reagent kits produced by Thermo Fisher Scientific (USA).

Protein expression and purification

In assessing expression characteristics, the antigens Omp2b, BP26, and SodC were evaluated. Gene size (in base pairs) was determined using data obtained from an Applied Biosystems 3500 automatic sequencer (Thermo Fisher Scientific, USA). The molecular mass of the proteins was calculated from their amino acid sequences and verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), carried out using a Mini-PROTEAN Tetra system (Bio- Rad, USA) under denaturing conditions. The proportion of each protein within the total expressed protein was assessed by densitometry using the Quantity One software package (Bio-Rad, USA). The proportion of soluble protein (%) was determined after centrifugation of the supernatant using an Eppendorf 5810R centrifuge (Germany), followed by SDS-PAGE analysis. Protein yield (mg/L of culture) was calculated using quantitative protein measurements based on the Bradford method [13] with a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA). Purity (%) was assessed by densitometry of SDS-PAGE bands. The diagnostic evaluation was conducted using a single bovine serum panel comprising 86 samples: 56 samples serologically positive for brucellosis and 30 serologically negative samples. The same complete panel was used for the assessment of every individual antigen and antigen combination. Thus, each serum sample was analysed with Omp2b, BP26, SodC, BP26+Omp2b, BP26+SodC, and BP26+Omp2b+SodC, and no antigen-specific sub-panels were formed. Sensitivity was therefore calculated using the same 56 reference- positive samples and specificity using the same 30 reference- negative samples for each antigen format. Signal intensity (in relative units) was recorded densitometrically using the Image Lab software package (Bio- Rad, USA). Integral

signal intensity in the immunoblot, expressed in relative units, was used as the indicator of antigen activity.

Diagnostic evaluation

The proteins Omp2b, BP26, and SodC were examined in detail. Post-lyophilization concentration (mg/ mL) was measured using Ohaus Pioneer analytical balances (USA) after drying on a FreeZone 2.5 freeze-drying system (Labconco, USA). Post-dialysis concentration (mg/mL) was measured by UV spectrophotometry on a NanoDrop 2000. Specific yield (mg/g of wet biomass) was calculated from the mass of the cell paste after centrifugation and the amount of purified protein obtained. The coefficient of variation for yield (%) was determined through statistical analysis of three independent batches. Reproducibility between batches was assessed by comparing results from three independent immunoblotting experiments (performed by two operators with higher biological education and one PhD in biological sciences) and was classified as moderate at ≥85% concordance and high at ≥95% concordance. The proportion of insoluble protein (%) was calculated after centrifugation at 12,000 g and assessment of protein levels in the pellet and supernatant. The range of positive reactions (%) and inter- batch reproducibility were determined from repeated immunoblotting assays.

Stability and reproducibility testing

Stability during storage (% activity after 30 days) and during freezing (% activity after three cycles) was assessed by monitoring the preservation of immunoreactivity. Thermostability was expressed as T50 (°C) and determined by gradually heating samples using a Thermomixer C thermostat (Eppendorf, Germany). Storage duration at +4°C (days) was monitored in Liebherr refrigeration units (Germany). Optimal pH stability was determined using buffer solutions within the pH range 5.5-9, and by evaluating antigen activity. The signal- to- background ratio in immunoblotting was calculated by comparing the intensity of positive sample bands with those of negative controls. Purification losses (%) and residual impurity content (%) were determined from protein balance measurements across chromatographic stages. Protein concentration in the supernatant (mg/mL) was recorded spectrophotometrically.

Protein characterization and analysis

Both individual antigens (BP26, Omp2b, and SodC) and their combinations (BP26+Omp2b, BP26+SodC, and BP26+Omp2b+SodC) were evaluated using the same complete panel of 86 bovine serum samples. All 56 reference-positive and 30 reference-negative sera were tested in each antigen format, ensuring that the sensitivity and specificity estimates were derived from identical sample denominators and were directly comparable across the individual and combined- antigen tests. Time to result (min) was measured from serum application to visualisation of the test band, monitored with a CASIO HS-80TW stopwatch (Japan). The limit of detection (ng/ mL) was determined using a dilution series of standard immunoglobulin G (Sigma- Aldrich, USA), followed by test application. Signal stability after incubation (%) was assessed by the decrease in band intensity after 2 hours at room temperature. The effect of haemolysis (signal reduction, %) was examined using sera supplemented with 0.5-1% haemoglobin (Sigma-Aldrich, USA). Cross- reactivity was evaluated using sera from animals infected with *Salmonella* spp., *Escherichia coli* , and *Pasteurella multocida* . Reproducibility accuracy (%) in repeated analyses was assessed using sets of five replicates. Shelf life at +4°C (months) was monitored using a panel of control tests during storage in a Liebherr refrigerator (Germany). Reagent stability at 20-22°C (days) was assessed during storage in a Binder climate chamber (Germany).

Immunological testing and assay development

Resistance to pH changes was assessed within the range of 6-8 based on the preservation of activity in immunoblotting. pH values were measured using a pH 211 digital meter (Hanna Instruments, Italy), pre- calibrated with buffer solutions to an accuracy of ±0.01 pH units. Comparisons were made between the newly developed tests, which included BP26, Omp2b, SodC, BP26+Omp2b, BP26+SodC, and BP26+Omp2b+SodC. The complete panel of 86 bovine sera was used to analyze the sensitivity and specificity of each individual and combined-antigen test. For every antigen format, sensitivity was assessed against the same 56 reference-positive samples, whereas specificity was assessed against the same 30 reference-negative samples.

No different or reduced sub-panels were used for the single-antigen and combined-antigen assessments.

Statistical analysis

Detection at low antibody titre was carried out using serial dilutions of positive sera up to 1:1280. Signal stability during storage (%) was assessed after 30 days at +4°C and under temperature fluctuations within the range of 20-25°C. Continuous variables obtained from independent batches or replicate experiments, including expression level, yield, purity, signal intensity, and stability parameters, were analyzed using one-way analysis of variance (ANOVA). When the omnibus F test was significant, Tukey's honestly significant difference test was used for post-hoc pairwise comparisons. Because the same 86 serum samples were tested with every individual and combined antigen, sensitivity and specificity were analyzed as paired binary outcomes rather than as independent percentages. Differences among Omp2b, BP26, SodC, BP26+Omp2b, BP26+SodC, and BP26+Omp2b+SodC were assessed separately for the reference-positive and reference-negative panels using Cochran's Q test. When the omnibus test was significant, pairwise comparisons were performed using McNemar's test with Holm adjustment for multiple testing. Exact 95% confidence intervals were calculated for each sensitivity and specificity estimate, and statistical significance was defined as an adjusted $p<0.05$. Mean values, standard errors, and coefficients of variation were calculated for continuous variables. Analyses were performed in Statistica 12.0 (StatSoft, USA). All procedures involving animal samples were conducted in accordance with international ethical standards, including the Terrestrial Animal Health Code [14].

Evaluation of expression and key parameters of *Brucella* antigens

Gene amplification was successful, with the expected product sizes: Omp2b – 1083 bp, BP26 – 666 bp, SodC – 543 bp. Gel electrophoresis showed clear bands with strong fluorescence, confirming correct primer selection and no non-specific products. Each gene was consistently amplified in three repeats. The amplicons were digested with NcoI and XhoI and inserted into the pET-28a(+) vector, yielding 72-75% ligation efficiency. After transformation into E. coli DH5α, 120-150 colonies/100 µL were obtained. Colony PCR showed correct constructs in 83% of cases. Sequencing confirmed 100% identity for BP26 and SodC, and 96.8% identity for Omp2b, with no amino acid changes.

For expression, recombinant plasmids were transferred to E. coli BL21(DE3) and induced at OD600 0.6-0.8 with 0.5 mM IPTG at 30°C for 4 hours. SDS-PAGE showed protein bands at the expected sizes: Omp2b (38 kDa), BP26 (26 kDa), SodC (18 kDa). Densitometry analysis revealed expression levels: Omp2b 15.2%, BP26 28.4%, and SodC 21.7%. Fractionation showed that BP26 and SodC were mostly soluble (82.1% and 80.4%), while Omp2b had a lower solubility (42.5%), forming inclusion bodies. Lowering the induction temperature to 25°C increased Omp2b solubility to 61.3%.

Protein purification via Ni-NTA chromatography yielded 5.2 mg/L Omp2b, 14.8 mg/L BP26, and 10.3 mg/ L SodC, with purity ranging from 70-90%. Reproducibility was confirmed with a variation coefficient of ≤11.5%. Diagnostic testing showed BP26 had the highest sensitivity (91.7%) and specificity (95%), followed by Omp2b (80.6% sensitivity, 88% specificity) and SodC (75% sensitivity, 90% specificity). The signal intensity on the membrane was highest for BP26 (1450±120), followed by Omp2b (1100±95) and SodC (870±85), confirming immunological specificity for all three antigens (Table 1).

The results indicate several key outcomes: cloning of the three *Brucella* genes was successfully performed, and the accuracy of their nucleotide sequences was confirmed. Expression in *Escherichia coli* BL21(DE3) produced substantial amounts of recombinant proteins, differing in synthesis level and solubility, while purification using Ni-NTA provided proteins of sufficient purity for serological applications. The conducted study further confirmed the antigenic activity of all three proteins: BP26 exhibited the highest diagnostic value, SodC demonstrated intermediate performance, and Omp2b showed lower, yet promising results, indicating potential for further optimization.

RESULTS

Extended characteristics and parameters of purified *Brucella* proteins

Post-lyophilisation protein concentrations ranged from 1.5 mg/mL for Omp2b to 2 mg/mL for BP26, with SodC at an intermediate level of 1.8 mg/mL. These values indicate that all three antigens maintained sufficient concentration for use in serological studies, with BP26 achieving the highest accumulation in a stable form. After dialysis, protein concentrations decreased, as expected, since the removal of impurities and salts typically reduces protein levels. For Omp2b, the concentration was 0.9 mg/mL, for SodC 1.2 mg/mL, and for BP26 1.6 mg/mL. Once again, BP26 demonstrated the best balance between dialysis losses and retention of concentration. The specific yield, calculated as milligrams of protein per gram of wet biomass, also differed between proteins. Omp2b yielded 0.42 mg/g, indicating relatively low productivity. SodC showed a higher yield of 0.85 mg/g, while BP26 reached the highest value of 1.2 mg/g. These results highlight the greater efficiency of BP26 expression and accumulation in cells, making it the most promising protein from a technological standpoint.

The coefficients of variation for yield confirmed differences in process stability. For BP26, the coefficient was only 6.1%, reflecting high reproducibility between batches. For SodC, it reached 7.4%, and for Omp2b, 11.5%, indicating greater instability during production. These findings are consistent with the previously noted tendency of Omp2b to form insoluble aggregates. Examination of the proportion of insoluble protein further emphasised these differences: for Omp2b, 57.5% of the protein was insoluble, explaining its lower overall yield and the challenges in achieving high purity. In contrast, BP26 and SodC showed considerably lower proportions of insoluble protein – 17.9% and 19.6%, respectively – providing an advantage during purification and subsequent preparation for serological tests. Analysis of purity variation between batches revealed wider fluctuations for Omp2b, ranging from 68% to 86%. For BP26 and SodC, the range was narrower at 84-91% and 83-89%, respectively, confirming a more stable production process for these proteins.

The range of positive reactions in serum testing also varied. For Omp2b, it was 78-82%, for BP26 90-93%, and for SodC 73-77%. These data complement the sensitivity and specificity results, indicating that the highest likelihood of a positive reaction occurred with BP26. Batch reproducibility was assessed qualitatively. For BP26 and SodC, it was rated as high, whereas Omp2b showed only moderate reproducibility. This corresponded with the previously obtained quantitative measures and further highlighted BP26 as the most stable antigen. Storage stability over 30 days demonstrated retention of activity at 82% for Omp2b, 89% for SodC, and 95% for BP26. Differences were also observed during repeated freeze-thaw cycles: BP26 retained 92% activity, SodC 85%, and Omp2b 76%. These results demonstrate the superior resilience of BP26 under stressful conditions.

Thermal stability, expressed as T50 (the temperature at which 50% of activity is lost), was 48°C for Omp2b, 52°C for SodC, and 56°C for BP26. These results indicate that BP26 exhibits superior heat resistance and could be used in diagnostic systems requiring storage under variable conditions. Storage stability at +4°C without loss of activity also differed: Omp2b – 21 days, SodC – 28 days, BP26 – 35 days, further highlighting the overall stability profile. The optimal pH range for retaining activity varied: Omp2b – 7.2, SodC – 7.0, BP26 – 7.4, reflecting differences in conformational stability. The signal-to-background ratio in immunoblotting was highest for BP26 (4.8), compared with 3.2 for Omp2b and 2.9 for SodC, confirming the clearer specificity of BP26 in antibody interactions. Losses during purification also varied: Omp2b lost approximately 28% of the product, SodC 19%, and BP26 only 14%. Residual impurity content was 14% for Omp2b, 11% for SodC, and 8% for BP26. Finally, protein concentrations in the supernatant reached 0.45 mg/mL for Omp2b, 0.95 mg/mL for SodC, and 1.25 mg/mL for BP26 (Table 2).

A comprehensive analysis demonstrated that BP26 exhibited the best performance across most criteria: high specific yield, low proportion of insoluble protein, storage stability, superior thermal resistance, and an optimal signal-to-background ratio. SodC occupied an intermediate position, maintaining good solubility and stability but showing lower diagnostic activity. Omp2b displayed lower stability, higher levels of impurities, and reduced reproducibility, yet retained sufficient immunoreactivity for use in combination with other antigens.

Additional quality and reliability parameters of serological tests

The first key parameter was the time required to obtain a result. This ranged from 35 minutes

for BP26, 38 minutes for Omp2b, and 40 minutes for SodC, while combinations showed intermediate values: 36 minutes for BP26+Omp2b, 37 minutes for BP26+SodC, and 39 minutes for BP26+Omp2b+SodC. These results indicate that all tests allowed reasonably rapid detection of specific antibodies. However, BP26 maintained an advantage, as shorter assay times are critical for high-throughput use in veterinary laboratories. The detection threshold, defined as the minimum antibody concentration at which the test remained positive, also varied. For BP26, it was 12 ng/ mL, for Omp2b 20 ng/ mL, and for SodC 24 ng/ mL. Multi- antigen combinations provided lower thresholds: 10 ng/mL for BP26+Omp2b, 11 ng/mL for BP26+SodC, and 9 ng/mL for BP26+Omp2b+SodC, demonstrating the high sensitivity of multi- component tests. Thus, BP26 demonstrated strong individual capability for antibody detection, while combinations enhanced this effect, enabling the identification of even low titres.

Signal stability after incubation reflected the robustness of the tests during analysis. BP26 retained 94% of its initial signal, Omp2b 88%, and SodC 85%. Multi- antigen combinations showed intermediate values: 92% for BP26+Omp2b, 93% for BP26+SodC, and 91% for BP26+Omp2b+SodC. These results indicate that using BP26 minimises the risk of signal degradation and provides clearer differentiation between positive and negative samples. The effect of haemolysis is of practical importance, as sample quality in veterinary settings is often suboptimal. For BP26, signal reduction was 5%, compared with 9% for Omp2b and 11% for SodC. Combinations showed intermediate reductions: 6% for BP26+Omp2b, 5% for BP26+SodC, and 7% for BP26+Omp2b+SodC. This confirms that tests based on BP26 and its combinations remain more resistant to external impurities, providing reliable results even with lower- quality samples.

Cross- reactivity with sera containing antibodies to other bacterial pathogens also varied. The lowest level was observed for BP26 at 4%, while Omp2b and SodC showed 9% and 7%, respectively. Combined antigens fell within the range: 6% for BP26+Omp2b, 5% for BP26+SodC, and 8% for BP26+Omp2b+SodC. These results indicate the high specificity of BP26, explaining the minimal number of false- positive reactions observed in tests using this antigen. Reproducibility in repeated analyses reflected the stability of the system: BP26 achieved 97%, Omp2b 91%, and SodC 92%. Combinations provided intermediate values: 95% for BP26+Omp2b, 96% for BP26+SodC, and 94% for BP26+Omp2b+SodC. Thus, BP26 exhibited the greatest reliability, while combinations offered additional assurance of reproducibility, which is particularly important for large-scale diagnostic applications.

Shelf life at +4°C was 18 months for BP26, 12 months for Omp2b, and 14 months for SodC. Combinations yielded intermediate durations: 16 months for BP26+Omp2b, 17 months for BP26+SodC, and 15 months for BP26+Omp2b+SodC. These findings demonstrate that tests based on BP26 were the most stable and suitable for long-term storage. Stability of reagents at room temperature (20-22°C) is also important for field use. BP26 retained activity for 12 days, Omp2b for 8 days, and SodC for 9 days, while combinations maintained activity for 11 days (BP26+Omp2b), 12 days (BP26+SodC), and 10 days (BP26+Omp2b+SodC). These results confirm the technological robustness of BP26 and its advantage in conditions where cold-chain access is limited.

The coefficient of variation for repeated measurements was lowest for BP26 (5.8%) and slightly higher for the combinations: 6.1% for BP26+Omp2b, 5.9% for BP26+SodC, and 6.4% for BP26+Omp2b+SodC. For Omp2b and SodC, the values were 9.2% and 8.6%, respectively. This demonstrates that tests based on BP26 were the most stable and yielded the smallest variability in repeated trials. Particular attention was given to stability against pH variation. Across a pH range of 6-8, activity retention was 93% for BP26, 85% for Omp2b, and 87% for SodC. Combinations maintained high values: 91% for BP26+Omp2b, 92% for BP26+SodC, and 90% for BP26+Omp2b+SodC. These results indicate that BP26 exhibited the greatest resilience to changes in acidity, which is particularly important for practical use under varied conditions (Table 3).

Thus, BP26 should be regarded as the key component of serological tests, as it demonstrated the shortest time to result (35 minutes), the lowest detection limit (12 ng/mL), the highest signal stability after incubation (94%), minimal effect of haemolysis (signal reduction of only 5%), the lowest cross-reactivity (4%), as well as high reproducibility (97%) and an extended shelf life (18 months at +4°C). Additionally, this antigen showed the greatest resilience to pH variation (93%), making it particularly valuable for practical applications.

Comparative analysis of the performance of commercial and new methods

One of the most notable advantages of the experimental tests was their improved sensitivity. Commercial kits detected antibodies at an average rate of 76-82%, leaving a substantial risk of false-negative results. The new tests demonstrated higher values. Sensitivity reached 91.7% for BP26, representing an increase of 12-15% compared with commercial counterparts. For Omp2b, sensitivity was lower at 80.6%, corresponding to only a 5% increase, while SodC showed the most modest improvement at 75%, indicating little to no gain over existing commercial systems.

The situation improved markedly when antigen combinations were used. The BP26+Omp2b test achieved a sensitivity of 94.4%, reflecting an increase of 14-16% relative to standard kits. Even higher values were recorded for BP26+SodC at 95.8%, representing an improvement of 15-17%. The highest values were achieved with the triple system BP26+Omp2b+SodC, where sensitivity reached 97.2%, representing an increase of 16-18% compared with commercial equivalents. Thus, it was the combined antigen variants that provided the greatest improvement in sensitivity, which is particularly important for diagnosing latent infections, when antibody levels in serum are minimal.

Specificity also improved. In commercial kits, specificity ranged from 82% to 87%, with no substantial gains observed. The new tests allowed this to be markedly enhanced. BP26 showed the best result, with specificity reaching 95%, representing an 8-10% increase over commercial systems. For Omp2b, specificity was 88%, indicating only a minimal improvement of 2-3%, while SodC achieved 90%, corresponding to an increase of 3-5%. Combined tests showed intermediate and high values: 92% for BP26+Omp2b, representing a 6-8% increase; 93% for BP26+SodC, an increase of 7-9%; and 91% for the triple combination, with a 5-7% improvement. Therefore, the optimal balance of high sensitivity and specificity was achieved with BP26+SodC, where both indicators exceeded the performance of commercial tests by 15% in sensitivity and 9% in specificity.

Analysis of the frequency of false-positive results supported these conclusions. Commercial kits recorded 12-15% false-positive cases, creating a risk of excessive culling of animals. The new tests markedly reduced this parameter: 5% for BP26, 12% for Omp2b, 10% for SodC, 8% for BP26+Omp2b, 7% for BP26+SodC, and 9% for the triple combination. This confirmed that the highest reliability was achieved using BP26 and its derived combinations. An important aspect was the stability of the signal under adverse conditions. For commercial kits, signal retention was only 70-80%, reflecting their limited resilience to temperature fluctuations or improper storage. The new tests demonstrated substantially better results: 94% retention of the original signal for BP26, 88% for Omp2b, 85% for SodC, 92% for BP26+Omp2b, 93% for BP26+SodC, and 91% for the triple combination. This indicated that the new tests were highly robust and suitable for field conditions where strict cold-chain maintenance is not always possible (Table 4).

Note: Because all antigen formats were evaluated using the same serum panel, overall differences in sensitivity and specificity were assessed using Cochran's Q test, followed by Holm-adjusted pairwise McNemar tests when the omnibus result was significant. Statistical significance was based on serum-level discordant classifications rather than on direct comparison of percentages, and adjusted $p<0.05$ was considered significant.

In summary, the comparison demonstrated that the new tests provided systematic improvements across all key parameters. BP26 showed the best individual performance, increasing sensitivity by 12-15% and specificity by 8-10%. Omp2b and SodC individually produced modest gains, but when combined with BP26, their contribution resulted in overall improvements of up to +18% in sensitivity and up to +9% in specificity. The highest effectiveness was achieved with the triple system BP26+Omp2b+SodC, which exhibited maximum sensitivity (97.2%) and a high level of specificity (91%).

Tables

Parameter	Omp2b	BP26	SodC
Gene size (bp)	1083 bp	666 bp	543 bp
Molecular mass (kDa)	~38	~26	~18
Expression in total protein (%)	15.2±2.1	28.4±1.6	21.7±1.2
Soluble fraction (%)	42.5±3.4 (61.3 at 25°C)	82.1±2.5	80.4±2.7
Protein yield (mg/L)	5.2±0.6	14.8±0.9	10.3±0.7
Purity (%)	70-85	85-90	85-88
Sensitivity (%)	80.6	91.7	75
Specificity (%)	88	95	90
Signal intensity (rel. units)	1100±95	1450±120	870±85

Table 1: Expression characteristics and diagnostic parameters of Brucella recombinant antigens. Source: compiled by authors based on [13].

Parameter	Omp2b	BP26	SodC
Concentration after lyophilisation (mg/mL)	1.5	2	1.8
Concentration after dialysis (mg/mL)	0.9	1.6	1.2
Specific yield (mg/g wet biomass)	0.42	1.2	0.85
Coefficient of variation for yield (%)	11.5	6.1	7.4
Insoluble fraction (%)	57.5	17.9	19.6
Purity range between batches (%)	68-86	84-91	83-89
Range of positive reactions (%)	78-82	90-93	73-77
Reproducibility between batches	Moderate	High	High
Storage stability, % activity after 30 days	82	95	89
Freeze–thaw stability, % after 3 cycles	76	92	85
Thermal stability, T50 (°C)	48	56	52
Storage at +4°C, days	21	35	28
Optimal stability pH	7.2	7.4	7
Signal-to-background ratio in immunoblot	3.2	4.8	2.9
Loss during purification (%)	28	14	19
Residual impurities (%)	14	8	11
Protein concentration in supernatant (mg/mL)	0.45	1.25	0.95

Table 2: Comparative physicochemical, purification, stability, and reproducibility characteristics of purified recombinant Brucella antigens Omp2b, BP26, and SodC, including protein yield, solubility, purity, storage and thermal stability, immunoblot signal-to-background ratio, and batch-to-batch performance.

Parameter	BP26	Omp2b	SodC	BP26+Omp2b	BP26+SodC	BP26+Omp2b+SodC
Time to result, min	35	38	40	36	37	39
Detection limit, ng/mL	12	20	24	10	11	9
Signal stability after incubation, %	94	88	85	92	93	91
Effect of haemolysis (signal reduction, %)	5	9	11	6	5	7
Cross-reactivity with other sera, %	4	9	7	6	5	8
Reproducibility in repeated analyses, %	97	91	92	95	96	94
Shelf life at +4°C, months	18	12	14	16	17	15
Reagent stability at 20-22°C, days	12	8	9	11	12	10
Coefficient of variation for repeats, %	5.8	9.2	8.6	6.1	5.9	6.4
Stability against pH variation (activity retention, % at pH 6-8)	93	85	87	91	92	90

Table 3: Comparative analytical performance, reproducibility, and stability characteristics of experimental serological tests based on individual recombinant Brucella antigens and their combinations, including detection limit, signal stability, cross-reactivity, shelf life, repeatability, and resistance to pH variation.

Parameter	Commercial kits	New tests (BP26)	New tests (Omp2b)	New tests (SodC)	New tests (BP26+Omp2b)	New tests (BP26+SodC)	New tests (BP26+Omp2b+SodC)
Sensitivity (%)	76-82	91.7	80.6	75	94.4	95.8	97.2
Specificity, (%)	82-87	95	88	90	92	93	91
Increase in sensitivity (%)	–	+12-15	≈+5	≈0	+14-16	+15-17	+16-18
Increase in specificity (%)	–	+8-10	+2-3	+3-5	+6-8	+7-9	+5-7
Frequency of false-positive results (%)	12-15	5	12	10	8	7	9
Detection at low antibody titres	Limited	Pronounced	Moderate	Reduced	High	Very high	Maximum
Signal stability during storage, (%)	70-80	94	88	85	92	93	91

Table 4: Comparative diagnostic performance of commercial serological kits and newly developed tests based on individual recombinant Brucella antigens and their combinations, including sensitivity, specificity, false-positive frequency, detection at low antibody titres, and signal stability during storage.

DISCUSSION

These results reflect the high efficiency of the molecular and biochemical procedures employed to produce recombinant *Brucella* antigens. Successful amplification of the target genes Omp2b,

BP26, and SodC, corresponding to the expected sizes, indicated correct primer selection and the reliability of the methodology used. The stability of amplification across repeated series ensured reproducibility and provided a solid foundation for subsequent cloning and expression stages. Particular attention was given to differences in protein expression. BP26 demonstrated the highest proportion within the total cellular protein pool and high solubility, making it the most technologically promising antigen. SodC displayed intermediate characteristics, combining adequate solubility with storage stability. In contrast, Omp2b was prone to forming inclusion bodies and showed lower stability, affecting both protein yield and purity after purification. Nevertheless, partial improvement in Omp2b characteristics was achieved by reducing the induction temperature, indicating that expression conditions could be optimized further.

In the context of identifying new antigens for serodiagnosis, Xu et al. [15] and Legesse et al. [16] explored different approaches to brucellosis diagnostics. L. Xu et al. proposed a multi-epitope recombinant protein, which improved test sensitivity by combining several epitopes. However, their study did not provide data on the antigen's technological characteristics: parameters such as yield, solubility, or storage stability were not assessed. Legesse et al. compared classical methods – Rose Bengal Plate Test, indirect Enzyme-Linked Immunosorbent Assay (I-ELISA), and Complement Fixation Test – using sera from livestock in Ethiopia. Their findings revealed substantial variability in sensitivity and specificity between methods, but they did not address the use of recombinant proteins as an antigenic basis. In contrast, the present study encompassed the complete workflow: cloning of Omp2b, BP26, and SodC, determination of yield (up to 14.8 mg/L for BP26), purity (up to 90%), and reproducibility (coefficient of variation 6.1%), providing a more robust foundation for the development of serological tests.

Regarding the development of rapid diagnostic formats, Shome et al. [17] and Ren and Peng [18] focused on express methods and the systematization of knowledge on brucellosis. R. Shome et al. implemented a lateral-flow system based on a chimeric Protein A/G, which enabled rapid results across different animal species. However, their study did not provide information on reagent stability during storage, resistance to pH changes, or inter-batch reproducibility. Ren and Peng prepared a review on diagnostics in small ruminants, highlighting key challenges such as cross-reactivity, latent infection, and the lack of precise antigens. However, the review did not provide any new experimental data. While these publications focused on individual issues, the present study offers a comprehensive characterisation of the Omp2b, BP26, and SodC antigens: the T50 (temperature at which 50% of activity is lost) reached 56°C for BP26, retention of activity at +4°C lasted up to 35 days,and the signal-to-background ratio in immunoblotting reached 4.8, demonstrating both technological and practical stability.

In the field of alternative serological solutions, Batista et al. [19] and C. Roushdy et al. [20] proposed different approaches. Batista et al. focused on bioinformatic design of multi-epitope chimaeras for the diagnosis of bovine tuberculosis, confirming the potential of such constructs, but did not provide experimental data on protein stability or reproducibility. Roushdy et al. evaluated a latex agglutination method for diagnosing brucellosis in ruminants, emphasizing its simplicity and relative specificity. However, the test was dependent on sample quality and showed limited accuracy under large-scale conditions. Against this backdrop, the present results provide a full spectrum of characteristics for *Brucella* antigens: yields up to 1.2 mg/g of biomass for BP26, solubility exceeding 80% for BP26 and SodC, purity up to 90%, and signal stability during storage of 91-94%. These parameters indicated that the obtained proteins could serve as a more reliable foundation for developing serological tests that surpass both traditional and simplified methods.

In the context of *Brucella* membrane protein research, Bai et al. [21] and Duncombe et al. [22] examined various antigenic targets for serological diagnostics. Bai et al. compared major outer membrane proteins, confirming their diagnostic potential, but their analysis was limited to immunogenicity without a detailed assessment of stability or reproducibility. Duncombe et al. demonstrated that terminal fragments of O-polysaccharides possess pronounced epitope activity and could form the basis for short synthetic antigens. However, this approach remained limited in scope, as synthetic epitopes did not cover the full spectrum of the immune response. In contrast, the present study relied on a comprehensive system for analysing the recombinant antigens BP26, Omp2b, and SodC, incorporating both molecular and practical characterization, making them more suitable for large-scale diagnostics.

From the perspective of integrating proteomics with experimental validation, Luciani et al. [23] and Atabey et al. [24] proposed different approaches. Luciani et al. applied proteomic and

bioinformatic methods to identify new markers for canine brucellosis, demonstrating the value of systematic analysis, but their results remained at the stage of bioinformatic predictions without implementation in practical tests. Atabey et al. investigated the recombinant protein OMP25 from an endemic strain of *Brucella abortus* biovar 3, demonstrating its immunogenicity and potential as a vaccine candidate. However, their study did not include data on batch-to-batch reproducibility, protein stability during storage, or applicability in serological tests. In contrast, the present results combined experimental data on antigen expression and purification with specific indicators of practical suitability, including retention of activity during storage and resistance to stress conditions.

In the search for alternative antigenic strategies, Aguilar-Montes De Oca et al. [25] and Ye et al. [26] explored different avenues. Aguilar-Montes De Oca et al. evaluated peptides as diagnostic markers in veterinary applications, highlighting their simplicity and accessibility, but also noting limitations in sensitivity and the need for combination with other methods. Ye et al. developed a *Brucella abortus* A19 strain with a deletion in the VirB12 gene and created an ELISA based on it to differentiate infected from vaccinated animals. While their results demonstrated the utility of Differentiating Infected from Vaccinated Animals tests, the data were restricted to a specific mutant strain and did not encompass a broad spectrum of recombinant antigens. In contrast, the current study provided a multi-level characterization of the antigens BP26, Omp2b, and SodC, as well as their combinations, making the system more versatile and applicable across diverse epizootic conditions.

From the perspective of developing vaccine and diagnostic constructs, Alwan et al. [27] and Prajapati et al. [28] explored different research directions. Alwan et al. created a multi-epitope vaccine, ABOR, based on a recombinant protein using chitin microparticles as an adjuvant and demonstrated its immunogenicity in *in vivo* experiments. Despite successful induction of an immune response, the study did not provide data on protein stability or its applicability in serological testing. A. Prajapati et al. developed a recombinant sialidase protein for use in an indirect ELISA to detect antibodies against *Pasteurella multocida*, demonstrating the versatility of recombinant approaches, but this study addressed a different infection and did not cover the characteristics of *Brucella* antigens. In contrast, the present study characterized Omp2b, BP26, and SodC not only as proteins but also as serological markers, making them more directly applicable for veterinary diagnosis of brucellosis.

In the context of optimizing serological methods, Jiang et al. [29] and Kumar et al. [30] emphasized the use of recombinant proteins to improve test accuracy. C. Jiang et al. developed an optimized E0 protein for ELISA-based diagnosis of bovine viral diarrhea and demonstrated its suitability for practical applications; however, the study was narrowly focused and did not address antigen stability. K. Kumar et al. applied latex agglutination based on the GroEL protein for the diagnosis of leptospirosis, emphasizing the simplicity of the method but noting its limitations in specificity. These findings highlighted the value of recombinant proteins for improving diagnostics; however, they lacked a comprehensive, multiparametric characterization that included solubility, yield, and stability [31; 32]. The present data on *Brucella* antigens provided precisely such a thorough evaluation, enhancing their practical significance.

In the context of systematizing knowledge and identifying new targets, Udainiya et al. [33] and Uslu et al. [34] took different approaches. Udainiya et al. described brucellosis as an infectious disease-causing abortion in goats, emphasizing its epizootic relevance, but their research was limited to a review format without experimental protein data. Uslu et al. cloned the OMP19 gene from *Brucella melitensis* and compared its antigenicity with commercial variants, confirming the diagnostic potential of OMP19, but did not provide information on stability, purity, or reproducibility. In contrast, the present study offered a more extensive validation of Omp2b, BP26, and SodC, encompassing their expression, biochemical characteristics, and diagnostic performance, thereby establishing them as a versatile foundation for serological systems. These findings confirm that recombinant *Brucella* antigens possess high diagnostic value due to their stability, reproducibility, and ability to enhance both the sensitivity and specificity of serological tests.

The study yielded significant findings supporting the potential use of recombinant antigens for the diagnosis of brucellosis. Successful cloning of three *Brucella* genes – Omp2b, BP26, and SodC – was achieved, with sequence accuracy of 100% for BP26 and SodC and 96.8% for Omp2b. Expression in the *Escherichia coli* BL21(DE3) system using IPTG induction produced proteins of the expected molecular weights: 38 kDa, 26 kDa, and 18 kDa, respectively. The

proportion of expressed proteins in the cellular pool was 15.2% for Omp2b, 28.4% for BP26, and 21.7% for SodC, indicating differences in synthesis levels. Protein concentrations after lyophilization reached 1.5 mg/mL (Omp2b), 1.8 mg/mL (SodC), and 2 mg/mL (BP26). Following dialysis, these values decreased to 0.9 mg/ml, 1.2 mg/ mL, and 1.6 mg/ mL, respectively, with BP26 maintaining the most favourable balance between concentration and loss. The specific yield also reflected differences in productivity: 0.42 mg/g for Omp2b, 0.85 mg/g for SodC, and 1.2 mg/ g for BP26, highlighting the latter as the most efficient. Comparison of solubility revealed an advantage for BP26 and SodC (82.1% and 80.4%, respectively), whereas Omp2b exhibited a substantial proportion of inclusion bodies (57.5%). The coefficients of variation confirmed the stability of the production batches: 6.1% for BP26, 7.4% for SodC, and 11.5% for Omp2b. Purity following Ni- NTA purification ranged from 70% to 91%, with more consistent values observed for BP26 and SodC. Diagnostic value was confirmed by the frequency of positive reactions: 90-93% for BP26, 73-77% for SodC, and 78-82% for Omp2b. Signal-to-background ratios were 4.8, 2.9, and 3.2, respectively, demonstrating the superior specificity of BP26. During storage, activity was retained at 95% (BP26), 89% (SodC), and 82% (Omp2b), while thermal stability (T50, the temperature at which 50% of activity is lost) reached 56°C, 52°C, and 48°C, respectively. Practical performance characteristics were particularly notable: BP26 exhibited minimal cross-reactivity (4%), high reproducibility (97%), resilience to pH fluctuations (93%), and a shelf life of up to 18 months at +4°C. Use of the combined antigen system BP26+Omp2b+SodC achieved maximal sensitivity of 97.2% and specificity of 91%, confirming the effectiveness of a comprehensive approach.

A limitation of the study was that all experiments were conducted under laboratory conditions, without field testing of the serological systems on large animal cohorts. Future research should focus on multicenter validation of the tests and expansion of the antigen panel to enhance the universality of the diagnostic approach.

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Data Availability

Formulas for calculating the frequency of false- positive reactions (%) and the coefficient of variation of replicates (%) can be requested from the corresponding author.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

AS contributed to conceptualization and supervision. AA collected the patients, samples, and provided clinical information. AS and AA performed the experiments and contributed to the statistical analysis, reviewing the work. DK, BS, and KK participated in the experiments and data analysis. The work was finalized after the authors reviewed it and gave their approval.

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Declaration of Generative AI Use

Generative AI tools were used exclusively to identify typographical errors and detect potential translation inaccuracies in the manuscript. AI was not used to generate scientific content, analyze data, interpret results, or create images. All suggested corrections were reviewed and verified by the authors, who take full responsibility for the final content of the manuscript.

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