

# Evaluation of the Sensitivity and Specificity of a Domestically Produced ELISA Kit for the Diagnosis of Bovine Brucellosis and Its Comparison with Common Serological and Molecular Tests

Maryam Akrami

Department of Biotechnology, Aria Vaccine Alborz Company (AVA)

Meisam Akrami

`mei.akrami.sci@iauctb.ac.ir`

Department of Biology, C.T.C., Islamic Azad University

---

## Research Article

**Keywords:** Domestically produced ELISA kit, Rose Bengal, 2-ME, Wright, Brucellosis, I-ELISA

**Posted Date:** September 21st, 2026

**DOI:** <https://doi.org/10.21203/rs.3.rs-10906633/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

**Additional Declarations:** No competing interests reported.

---

# Abstract

Brucellosis is a zoonotic disease of substantial public-health importance, owing to the widespread human consumption of animal products. Accurate and timely diagnosis is therefore a cornerstone of the control and prevention programmes administered by veterinary authorities. In Iran, routine diagnosis relies on the Rose Bengal Plate Test (RBPT) together with the complementary Wright (tube agglutination) and 2-mercaptoethanol (2-ME) assays. In many developed countries, the Wright and 2-ME tests have been superseded by more advanced techniques because they are laborious, time-consuming, and difficult to interpret. The enzyme-linked immunosorbent assay (ELISA) is among the most suitable alternatives: its high sensitivity and specificity, together with its ease of use, have prompted its adoption within national Brucellosis control and eradication programmes. As the Razi Vaccine and Serum Research Institute has recently succeeded in manufacturing a licensed domestic ELISA kit, the present study evaluated the diagnostic performance of this indirect ELISA (I-ELISA) relative to the Wright and 2-ME tests as reference standards. A total of 250 fresh bovine serum samples, randomly selected from commercial dairy herds in Alborz province, were tested by RBPT, the complementary assays, and the domestic ELISA kit. Using a composite reference standard (Wright/2-ME), the diagnostic sensitivity of the I-ELISA and RBPT were 96.8% and 100.0%, and their specificities were 100.0% and 92.7%, respectively. The assay demonstrated nearly perfect agreement with the reference standard (Cohen's Kappa = 0.989) and excellent repeatability (CV < 9%). To corroborate the molecular detection limits, PCR assays targeting *omp-2* and *16S rRNA* were also evaluated. The findings demonstrate that the domestically produced I-ELISA offers higher specificity than RBPT and exceptional concordance with confirmatory tests. It is additionally cost-effective, applicable in all laboratory settings, and free of operator-dependent interpretation, making it a highly reliable and viable replacement for conventional diagnostic methods in endemic regions.

## Introduction

Brucellosis remains one of the most economically and clinically significant zoonoses worldwide, and its human form—Malta fever—is epidemiologically tethered to the prevalence of infection in livestock reservoirs. The disease is sustained by a tightly host-adapted genus of pathogens, with five species of principal medical and veterinary relevance: *Brucella melitensis* (goats, sheep, cattle, and camels), *Brucella abortus* (cattle), *Brucella suis* (pigs), *Brucella canis* (dogs), and *Brucella maris* (marine mammals). The causative organism is a Gram-negative coccobacillus that is non-spore-forming, non-motile, and non-encapsulated. A defining feature of its pathogenesis is its capacity for facultative intracellular survival: by evading the bactericidal machinery of professional phagocytes and replicating within a membrane-bound vacuole, *Brucella* establishes persistent infection that is poorly accessible to humoral immunity and frequently progresses to a chronic, low-grade state. This intracellular tropism underlies both the relapsing clinical course in humans and the protracted shedding that perpetuates transmission within herds (1–3).

In Iran, Brucellosis has long been endemic, imposing a continuous burden through abortion and reduced fertility in livestock and through Malta fever in exposed human populations. The diagnosis of Brucellosis has consistently posed a formidable clinical and laboratory challenge, and it remains an active focus of research owing to the insidious onset and heterogeneous manifestations of the disease. After infection, the clinical picture evolves over time, and the short-lived, incomplete immunity that develops permits re-infection with variable presentations. In settings of high herd prevalence and among occupationally exposed groups—abattoir workers, herders, veterinarians, and laboratory personnel—infection frequently runs a mild and sometimes subclinical course, such that affected individuals neither seek medical attention nor receive definitive treatment, instead resorting to symptomatic self-medication. This clinical ambiguity places a disproportionate diagnostic burden on the laboratory, where the choice of assay critically determines whether infected hosts are correctly identified and removed from the transmission cycle (4–6).

A spectrum of laboratory methods is available for diagnosis, comprising serological assays—the Rose Bengal Plate Test (RBPT), the Wright tube agglutination test, the 2-mercaptoethanol (2-ME) test, and the enzyme-linked immunosorbent assay (ELISA)—and molecular techniques such as the polymerase chain reaction (PCR). Each modality interrogates a distinct facet of the host–pathogen interaction and, consequently, occupies a different position along the trade-off between diagnostic sensitivity and specificity. The agglutination-based tests detect anti-smooth-lipopolysaccharide (S-LPS) antibodies and, while inexpensive and rapid, are vulnerable to cross-reactivity with other Gram-negative organisms that share O-polysaccharide epitopes (notably *Yersinia enterocolitica* O:9), to prozone phenomena, and to operator-dependent endpoint interpretation. The 2-ME test refines this approach by chemically dissociating IgM pentamers, thereby isolating the IgG-dominated response that signals established or chronic infection; however, like the Wright test, it loses sensitivity in long-standing cases in which agglutinating titres wane (7–10).

At the molecular level of antigen recognition, a diverse repertoire of protein and polysaccharide antigens has been characterised across the outer membrane, inner membrane, cytoplasm, and periplasmic space of *Brucella*. These antigens have been resolved principally by electrophoretic and ELISA-based techniques; several are already exploited in human and veterinary diagnostics, while others have been advanced as candidate targets for higher-resolution platforms such as ELISA. Among them, the smooth LPS remains the immunodominant and most widely validated serodiagnostic antigen, whereas defined outer-membrane proteins offer prospects for improved specificity. Although radioimmunoassay and immunofluorescence were historically employed to characterise the antibody response, indirect ELISA (I-ELISA) has since become the reference approach for resolving immunoglobulin isotypes. I-ELISA is particularly well suited to the quantification of IgG, and an LPS-based indirect format can substitute for several of the conventional agglutination assays while furnishing an objective, instrument-read endpoint that eliminates inter-observer variability and lends itself to high-throughput, standardised screening (11–13).

While ELISA methodologies rely on well-established S-LPS antigens, the validation of an indigenously manufactured kit holds profound epidemiological and strategic significance. In endemic regions facing economic constraints, fragile supply chains, or international sanctions, reliance on imported diagnostic kits cripples continuous surveillance and national eradication programs. Therefore, validating a domestically produced I-ELISA under field-representative conditions goes beyond routine benchmarking; it ensures self-reliance in biosecurity and provides a scalable model for other developing nations striving to achieve FAO/WHO Brucellosis control targets. We hypothesised that this domestic I-ELISA would achieve diagnostic metrics equivalent to reference standards. The present study was designed to rigorously evaluate this kit on a panel of bovine sera and to compare its sensitivity, specificity, and precision directly against RBPT, using the Wright and 2-ME tests as a composite reference standard, with PCR serving as molecular corroboration (14–16).

## Materials and Methods

### Study Design and Sample Collection

A total of 250 fresh bovine serum samples were obtained from the Brucellosis Department of the Razi Vaccine and Serum Research Institute (Karaj, Iran). These samples were randomly selected from a larger pool of 1,250 dairy cows residing in commercial dairy farms in Alborz province, a region with a history of controlled endemic brucellosis. All sera were aliquoted and stored at  $-20^{\circ}\text{C}$  until analysis. Blood cultures and residual specimens were maintained at  $4^{\circ}\text{C}$  prior to DNA extraction. The study was designed as a retrospective diagnostic accuracy evaluation in which each serum sample was tested in parallel by four serological methods, with PCR employed as an auxiliary molecular evaluation tool.

### Serological Assays

#### Rose Bengal Plate Test (RBPT)

Equal volumes (30  $\mu\text{L}$ ) of serum and Rose Bengal antigen (prepared from standard *B. abortus* strain S.99 cultured on *Brucella* agar for 48 h at  $37^{\circ}\text{C}$  under 5%  $\text{CO}_2$ , inactivated with 10% formalin, and containing 8% cell mass at acidic pH) were dispensed onto a white ceramic plate. The plate was rotated on a mechanical rotator for 4 minutes at room temperature. The presence of any visible agglutination was interpreted as a positive result.

#### Wright Standard Tube Agglutination Test

Serial two-fold dilutions of each serum sample were prepared in physiological saline from 1:20 to 1:2560. Briefly, 0.8 mL of diluent was placed in the first tube and 0.5 mL in each of the subsequent tubes. Serum (0.2 mL) was added to the first tube, mixed thoroughly, and 0.5 mL was transferred to the second tube; this serial transfer was continued through the series. An equal volume (0.5 mL) of standardised *B.*

*abortus* antigen (0.4% cell mass, pH 7.0) was then added to each tube. Tubes were incubated at 37°C for 20–24 hours. The titre was recorded as the reciprocal of the highest dilution showing at least 50% clarification ( $\geq 2 +$  agglutination).

## 2-Mercaptoethanol (2-ME) Test

The 2-ME test was performed in parallel with the Wright test using an identical dilution series (1:20 to 1:2560). Serum dilutions were prepared in 0.1 M 2-mercaptoethanol to dissociate IgM pentamers, thereby selectively detecting IgG agglutinins. Following addition of standardised antigen, tubes were incubated at 37°C for 1 hour and read under the same criteria as the Wright test.

## Indirect ELISA (I-ELISA)

The domestically produced I-ELISA kit (Razi Vaccine and Serum Research Institute, Karaj, Iran) was employed according to the manufacturer's standardised protocol. The kit utilises smooth lipopolysaccharide (S-LPS) antigen extracted from *B. abortus* strain S.99, coated onto 96-well polystyrene microtitre plates. Test sera were applied at a final dilution of 1:300, while positive and negative control sera were applied at 1:10. A total volume of 100  $\mu$ L per well was used, and incubation was carried out for 30 minutes at room temperature. Following washing steps, horseradish peroxidase (HRP)-conjugated anti-bovine IgG was added, and colour development was achieved with 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Optical density (OD) was read spectrophotometrically at 450 nm.

Results were expressed as the sample-to-positive (S/P) ratio:

$$S/P = \frac{OD_{sample} - OD_{negative\ control}}{OD_{positive\ control} - OD_{negative\ control}}$$

Interpretation was performed according to the following thresholds:  $S/P \leq 50$  (negative),  $S/P 51-109$  (suspect), and  $S/P \geq 110$  (positive). An OD-based operational cut-off of 135 was additionally determined by ROC analysis as described below.

## DNA Extraction and PCR Amplification

Genomic DNA was extracted from *Brucella* cultures and serum samples using a commercial extraction kit (Bioneer, Germany) following the manufacturer's protocol. Two target loci within the *Brucella* genome were selected for amplification:

- *omp-2* gene, encoding an outer-membrane protein, yielding a 193 bp fragment
- *16S rRNA* gene, encoding the small ribosomal subunit, yielding a 905 bp fragment

## Primer sequences:

- *omp-2* (193 bp): JPF (GCAAATCGCTGGCGCTTGCT), JPR (GAAGGCCGCGTTATTCGACC)
- *16S rRNA* (905 bp): F4 (GCTGCTTCAGCGTCGTTAT), R2 (CGGGCTTCCGCTTTATGG)

PCR amplification was performed in a final reaction volume of 25 µL. The optimised reaction mixture contained: 2.5 µL of 10× PCR buffer, 0.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 10 pmol of each primer, 1.25 U of Taq DNA polymerase, and 5 µL of template DNA (approximately 200 ng). Thermocycling was carried out on a calibrated thermocycler with the following programme: initial denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 63.9°C (*omp-2*) or 48.3°C (*16S rRNA*) for 1 min, and extension at 72°C for 1 min; with a final extension at 72°C for 5 min. Amplification products were resolved by electrophoresis on 1.5% (w/v) agarose gels stained with ethidium bromide and visualised under ultraviolet transillumination alongside a 100-bp DNA ladder.

## Analytical Sensitivity of PCR

The limit of detection (LoD) for each PCR system was determined by preparing ten-fold serial dilutions of extracted genomic DNA in two matrices: (i) sterile distilled water and (ii) *Brucella*-negative bovine serum. Each dilution was amplified in triplicate under the optimised conditions specified above, and the lowest DNA concentration yielding a visible band of the expected size was recorded as the LoD.

## Statistical Analysis

To determine diagnostic sensitivity and specificity and to perform the Receiver Operating Characteristic (ROC) analysis, a Composite Reference Standard (CRS) was employed. In the absence of culture results for all fresh samples, sera that tested strictly negative across established confirmatory serological assays (Wright and 2-ME) were operationally defined as true negatives. Conversely, samples exhibiting positive concordance across the confirmatory tests were classified as true positives. Sensitivity and specificity were computed using standard epidemiological formulae. The agreement between the I-ELISA and the composite reference standard was quantified using Cohen's Kappa coefficient ( $\kappa$ ). Additionally, the intra-assay and inter-assay coefficients of variation (CV%) were calculated to evaluate the precision and repeatability of the domestic kit. Youden's index ( $J = \text{Sensitivity} + \text{Specificity} - 1$ ) was computed to identify the threshold maximising combined sensitivity and specificity. The area under the ROC curve (AUC) and its associated 95% confidence interval (CI) and standard error (SE) were calculated. All statistical analyses were performed using SPSS version 22 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 9.

## Results

### Serological Screening of the 250-Sample Panel

Of the 250 fresh bovine serum samples screened, 47 (18.8%) were reactive by RBPT. Based on the composite reference standard, 31 samples were identified as true positives (positive by both the Wright test and the 2-ME test), while 219 samples were true negatives. The I-ELISA classified 30 samples as positive and 220 as negative.

Wright titres among positive samples ranged from 1:20 to 1:640 (median 1:160), and 2-ME titres were consistently lower, ranging from 1:20 to 1:160 (median 1:40), consistent with the IgG-restricted nature of the 2-ME readout. All 250 sera were unequivocally classified as positive or negative by the I-ELISA using the  $S/P \geq 110$  threshold; no samples fell within the suspect range ( $S/P$  51–109).

## Diagnostic Performance and Concordance

Diagnostic sensitivity and specificity were calculated against the Wright/2-ME composite reference standard for the 250 fresh samples (Table 1). The I-ELISA demonstrated a superior specificity of 100.0% while retaining an excellent sensitivity of 96.8% (correctly identifying 30 out of 31 true positive samples). The RBPT achieved perfect sensitivity (100.0%) but exhibited a lower specificity of 92.7%, yielding 16 false-positive results. Crucially, the agreement between the I-ELISA and the composite reference standard was nearly perfect, yielding a Cohen's Kappa coefficient of 0.989 ( $p < 0.001$ ).

Table 1  
Diagnostic performance of serological assays evaluated on fresh bovine serum samples (n = 250) using Wright/2-ME as the composite reference standard.

| Assay   | True Positive (n) | False Positive (n) | True Negative (n) | False Negative (n) | Sensitivity (%) | Specificity (%) |
|---------|-------------------|--------------------|-------------------|--------------------|-----------------|-----------------|
| I-ELISA | 30                | 0                  | 219               | 1                  | 96.8            | 100.0           |
| RBPT    | 31                | 16                 | 203               | 0                  | 100.0           | 92.7            |

## ROC Analysis and Assay Precision

The distribution of  $S/P$  ratios revealed two clearly separated populations: samples from negative animals clustered below a value of 120, whereas those from positive cases exhibited  $S/P$  ratios predominantly in the range of 150–255 (Fig. 1).

ROC analysis confirmed the excellent discriminatory capacity of the I-ELISA, yielding an AUC of 0.991 (95% CI: 0.975–1.000;  $p < 0.0001$ ) (Fig. 2). The OD-based cut-off of 135, determined by maximising Youden's index, provided clean separation of the two populations with no overlap. To assess the repeatability of the domestically produced I-ELISA, intra-assay and inter-assay coefficients of variation (CV) were determined. The assay demonstrated high precision, with an intra-assay CV of 6.89% and an inter-assay CV of 8.97%, both falling well below the acceptable threshold of 10%.

## PCR Optimisation and Analytical Sensitivity

Under the optimised conditions, both the *omp-2* (193 bp) and *16S rRNA* (905 bp) targets were amplified consistently and reproducibly from *Brucella* genomic DNA, with no non-specific amplification products observed (Fig. 3).

The *16S rRNA* system demonstrated markedly superior sensitivity, detecting as little as 40 fg of template DNA in distilled water and 4 pg in serum. The *omp-2* system was approximately 100-fold less sensitive, with detection limits of 4 pg in water and 40 pg in serum. Notably, the presence of the serum matrix reduced detection sensitivity by 10- to 100-fold for both targets, underscoring the inhibitory effect of serum components on PCR amplification efficiency.

## Comparative Serological Profiles

A sample-wise comparative analysis of all 47 RBPT-positive sera revealed distinct serological patterns: strongly reactive (+++) RBPT samples consistently tested positive by both Wright/2-ME and I-ELISA. Conversely, a subset of weakly (+) or moderately (++) RBPT-positive samples ( $n = 16$ ) were classified as negative by Wright/2-ME and I-ELISA, illustrating the higher sensitivity but lower specificity of RBPT as a primary screening tool.

## Epidemiological and Demographic Distribution

An analysis of the demographic and spatial data (Fig. 4) revealed distinct patterns of Brucellosis distribution across the seven evaluated counties in Alborz province, with Karaj recording the highest frequency of positive cases ( $n = 10$ ) proportional to its sample size. Demographic stratification indicated a significantly higher prevalence of infection in adult cattle ( $> 2$  years) at 15.3% (26/170), compared to younger animals ( $< 2$  years) at 6.25% (5/80). Furthermore, local and mixed bovine breeds exhibited a greater positivity rate (17.0%, 17/100) than their industrial Holstein counterparts (9.3%, 14/150).

## Discussion

Accurate diagnosis of bovine Brucellosis remains a critical challenge in veterinary medicine, necessitating diagnostic methods that are not only sensitive and specific but also rapid, cost-effective, and technically feasible for routine laboratory use. While bacterial culture is considered the definitive diagnostic standard, its practical application is limited by prolonged incubation periods and significant false-negative rates in both acute and chronic cases, particularly when antibiotic therapy has been initiated. Consequently, serological assays have become the cornerstone of Brucellosis surveillance and control programs worldwide (17, 18).

The present study comprehensively evaluated the diagnostic performance of a domestically developed indirect ELISA (I-ELISA) kit against conventional serological tests (Rose Bengal, Wright, and 2-Mercaptoethanol) and molecular methods (PCR) using a well-characterized panel of 250 fresh bovine sera from an endemic setting. Our findings demonstrate the superior diagnostic accuracy of the I-ELISA kit, with a sensitivity of 96.8% and a specificity of 100.0% when compared to the composite reference standard. The Receiver Operating Characteristic (ROC) analysis yielded an Area Under the Curve (AUC) of

0.991, and the assay demonstrated near-perfect statistical agreement (Cohen's Kappa = 0.989) with established confirmatory assays. The high concordance observed aligns with previous research advocating for ELISA-based assays. Jugapur et al. reported that ELISA exhibited higher sensitivity (90%) than both Rose Bengal and Wright tests in cattle sera, particularly at low antibody titers (1/80), endorsing its utility for screening purposes. Similarly, Ahouran et al. demonstrated that indirect ELISA achieved 92% sensitivity compared to Wright and 2-ME tests in bovine samples, reinforcing its reliability as a confirmatory test (15, 19).

The optimization of PCR conditions in this study was critical for achieving reproducible amplification. Although whole blood, buffy coat, or tissue samples are the preferred matrices for *Brucella* PCR due to the intracellular tropism of the pathogen, this study utilized serum to align with the available routine serological submissions. The observed 10- to 100-fold reduction in analytical sensitivity when using serum compared to distilled water underscores the dual challenge of low cell-free *Brucella* DNA concentrations and the presence of PCR inhibitors in blood derivatives. This finding scientifically reaffirms that while PCR is a powerful confirmatory tool, PCR on serum cannot replace serology for primary screening; rather, it serves as a highly specific auxiliary method (12, 16).

The epidemiological demographic data generated in this study (Fig. 4) further underscores the practical utility of the domestic I-ELISA in targeted field surveillance. The higher positivity rate observed in adult cattle (> 2 years) is consistent with the chronic nature of Brucellosis and the cumulative risk of environmental exposure over time. Additionally, the elevated prevalence in local and mixed breeds compared to Holsteins likely reflects discrepancies in vaccination coverage, biosecurity stringency, and herd management practices between traditional rural farms and industrial dairy operations. Utilizing a cost-effective, high-throughput domestic I-ELISA enables veterinary authorities to conduct continuous, large-scale screening specifically focused on these high-risk demographics, thereby facilitating a more efficient allocation of disease eradication resources.

The choice of *Brucella abortus* S-LPS as the coating antigen for our I-ELISA kit was strategic, ensuring alignment with the antigens used in standard Wright, 2-ME, and Rose Bengal tests (prepared from *B. abortus* strain 99.S). This harmonization facilitates the direct comparison and potential replacement of these conventional tests. The performance metrics of our kit compare favorably with international studies; for instance, Nielsen recommended I-ELISA as a reference test due to its 91% sensitivity and 98% specificity for detecting *Brucella* IgM and IgG antibodies [1]. Furthermore, the observed intra-assay CV of 6.89% and inter-assay CV of 8.97% confirm that the domestic kit maintains robust batch-to-batch repeatability and precision (5, 16).

Classical serological tests, while historically valuable, suffer from well-documented drawbacks including subjective interpretation, prozone phenomena, and reduced specificity in endemic areas due to cross-reacting antibodies from exposure to other Gram-negative bacteria. One limitation of the present study is that cross-reactivity with other Gram-negative bacteria sharing O-polysaccharide epitopes (notably *Yersinia enterocolitica* O:9) was not explicitly evaluated using known positive sera. However, because the

evaluated I-ELISA utilizes the standard *B. abortus* S.99 S-LPS, its cross-reactivity profile is theoretically comparable to conventional methods. Furthermore, the use of an IgG-specific conjugate in the I-ELISA mitigates some non-specific IgM-mediated cross-reactions commonly seen in agglutination tests. Our I-ELISA kit mitigates standard serological issues through objective spectrophotometric reading, quantitative antibody measurement, and the establishment of a validated, ROC-derived cut-off value. From an economic and epidemiological perspective, the local production of this kit significantly reduces the cost per test compared to imported commercial alternatives, providing a highly cost-effective solution for mass screening programs in developing nations. It curtails foreign currency expenditure, ensures a stable supply chain independent of international sanctions, and fosters regional biosecurity, supporting control and eradication programs as recommended by FAO/WHO (2, 5, 6, 8, 9).

## Conclusion

This study successfully developed and validated a high-performance, domestically produced indirect ELISA kit for the serodiagnosis of bovine Brucellosis. The kit demonstrated exceptional diagnostic accuracy, precision (CV < 9%), and near-perfect statistical agreement ( $\kappa = 0.989$ ) with conventional confirmatory assays. The rigorous optimization and validation process, conducted in accordance with OIE guidelines, confirms its reliability and suitability for routine laboratory use. Given its superior sensitivity, specificity, cost-effectiveness, and alignment with international standards, we strongly recommend the widespread adoption of this indigenous I-ELISA kit to enhance diagnostic precision and foster economic resilience in endemic regions.

## Declarations

## Conflict of Interest:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Ethical Approval:

The protocol for the collection of guinea pig samples was reviewed and approved by the Animal Ethics Committee of the Razi Vaccine and Serum Research Institute (RVSRI), Karaj, Iran (Ethics approval code: IR.RVSRI.REC.1398.107). All procedures were performed in accordance with the relevant ethical guidelines and regulations.

## Funding:

Not applicable.

# Author Contribution

MaA: contributed to data curation, formal analysis, original draft, investigation, writing—review & editing;  
MeA: contributed to conceptualization, methodology, validation, formal analysis, resources, data curation, writing—review & editing, supervision, project administration.

# Acknowledgement

We appreciate the support of the Research Council of Aria Vaccine Alborz (AVA) Company.

# Data Availability:

Data will be made available on request.

# References

1. Abdi-Liae Z, Soudbakhsh A, Jafari S, Tomaj HEK. Haematological manifestations of Brucellosis. *Acta Medica Iranica*. 2007;145–8.
2. Afonso C, Miller P, Grund C, Koch G, Peeters B, Selleck P, et al. Manual of diagnostic tests and vaccines for terrestrial animals. Paris: World Organization for Animal Health. 2012.
3. Al-Attas RA, Al-Khalifa M, Al-Qurashi AR, Badawy M, Al-Gualy N. Evaluation of PCR, culture and serology for the diagnosis of acute human brucellosis. *Annals of Saudi medicine*. 2000;20(3–4):224–8.
4. Araj GF. Update on laboratory diagnosis of human brucellosis. *International journal of antimicrobial agents*. 2010;36:S12–S7.
5. Assanzhanova N, Zhugunissov K, Chervyakova O, Ryskeldinova S, Akmyrzayev N, Sagymbayeva A, et al. Modern Strategies for Brucellosis Vaccination: From Traditional Approaches to Innovative Platforms. *Vaccines*. 2026;14(5):409.
6. Bahador A, Mansoori N, Esmaeili D, Amini Sabri R. Brucellosis: Prevalence and retrospective evaluation of risk factors in western cities of Tehran province, Iran. *Journal of bacteriology research*. 2012;4(3):33–7.
7. Bosilkovski M. Brucellosis: it is not only Malta! Zoonoses-Infections Affecting Humans and Animals: Focus on Public Health Aspects: Springer; 2014. p. 287–315.
8. Buzgan T, Karahocagil MK, Irmak H, Baran AI, Karsen H, Evirgen O, et al. Clinical manifestations and complications in 1028 cases of brucellosis: a retrospective evaluation and review of the literature. *International journal of infectious diseases*. 2010;14(6):e469–e78.
9. Colmenero J, Reguera J, Martos F, Sánchez-De-Mora D, Delgado M, Causse M, et al. Complications associated with *Brucella melitensis* infection: a study of 530 cases. *Medicine*. 1996;75(4):195–211.

10. Corbel MJ. Brucellosis in humans and animals: World Health Organization; 2006.
11. Dorneles EMS, Santana JA, Alves TM, Pauletti RB, Mol JPdS, Heinemann MB, et al. Genetic stability of *Brucella abortus* isolates from an outbreak by multiple-locus variable-number tandem repeat analysis (MLVA16). *BMC microbiology*. 2014;14(1):186.
12. Gaddafi MS, Jolayemi KO, Lawal H, Musawa IA, Garba B, Goni MD, et al. Brucellosis prevalence in Nigerian livestock: a systematic review and meta-analysis. *BMC Infectious Diseases*. 2026.
13. Godfroid J, Garin-Bastuji B, Saegerman C, Blasco J. Brucellosis in terrestrial wildlife. *Revue Scientifique et Technique Office International des Epizooties*. 2013.
14. Johny S, Merisko A, Whitman DW. Efficacy of eleven antimicrobials against a gregarine parasite (*Apicomplexa*: Protozoa). *Annals of Clinical Microbiology and Antimicrobials*. 2007;6(1):15.
15. Liu Z, Shi Y, Xue C, Yuan M, Li Z, Zheng C. Epidemiological and spatiotemporal clustering analysis of human brucellosis—China, 2019 – 2023. *China CDC Weekly*. 2025;7(4):130.
16. Lu P, Luo B, Wang Q, Wang L, Chen M, Jia J, et al. Progress in brucellosis immune regulation inflammatory mechanisms and diagnostic advances. *European Journal of Medical Research*. 2025;30(1):830.
17. Nielsen K. Diagnosis of brucellosis by serology. *Veterinary microbiology*. 2002;90(1–4):447–59.
18. Rehman S, Ullah S, Kholik K, Munawaroh M, Sukri A, Malik MIU, et al. A detailed review of bovine brucellosis. *Open veterinary journal*. 2025;15(4):1520.
19. Salama AA. Serological and molecular diagnosis of bovine brucellosis in Menoufia province. *Journal of Current Veterinary Research*. 2019;1(1):115–26.

## Figures

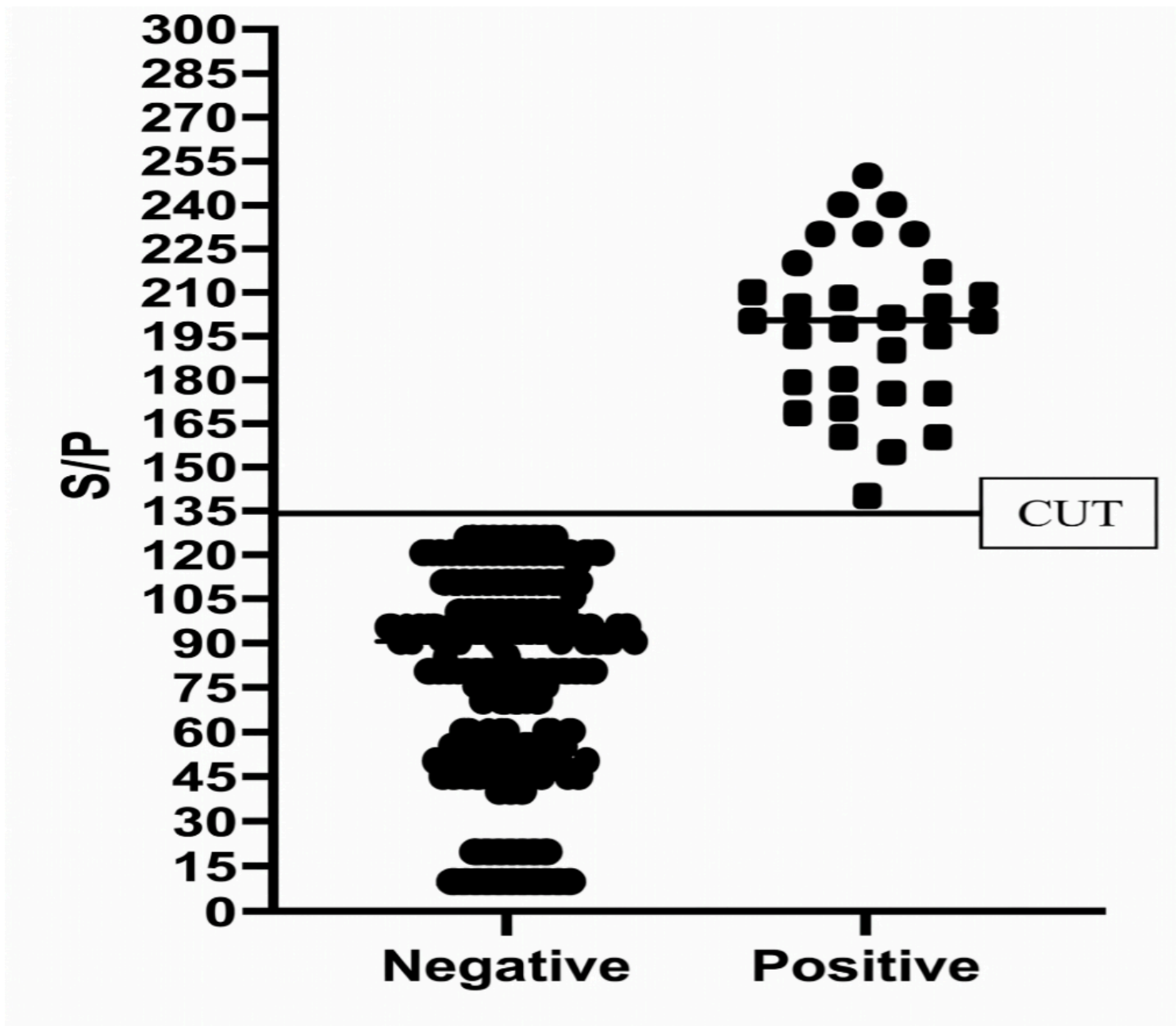


Figure 1

Distribution of sample-to-positive (S/P) ratios determined by the I-ELISA. The scatter plot illustrates the robust separation between the negative sera (circles) and true positive sera (squares). The horizontal solid line indicates the optimal diagnostic cut-off (S/P = 135) derived from the ROC analysis. Notably, only a single false-negative result is observed marginally below the cut-off threshold.

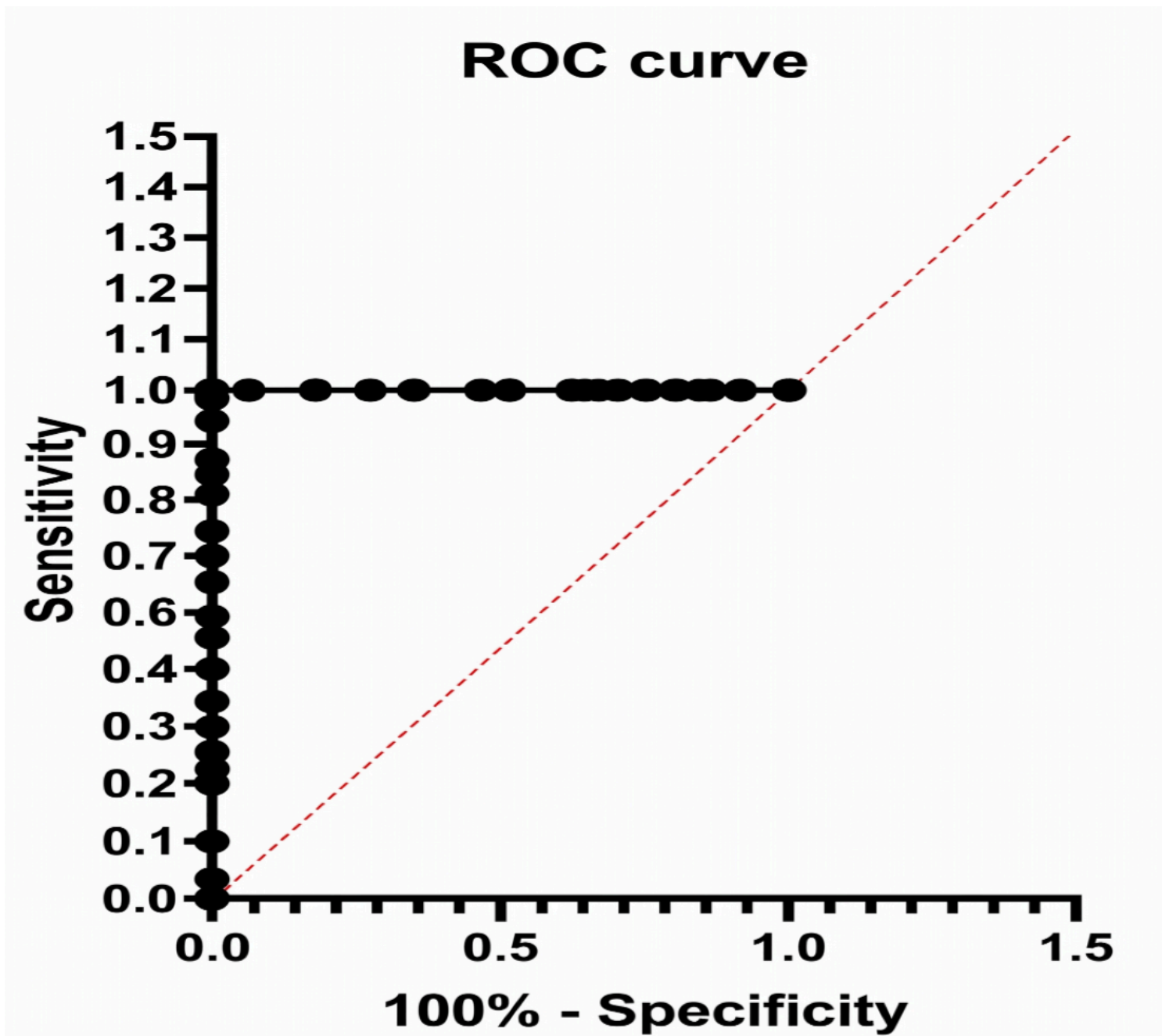


Figure 2

Receiver Operating Characteristic (ROC) curve analysis for the domestically produced I-ELISA evaluated against the composite reference standard (Wright/2-ME). The assay demonstrates excellent discriminatory capacity with an Area Under the Curve (AUC) of 0.991 (95% CI: 0.975–1.000).

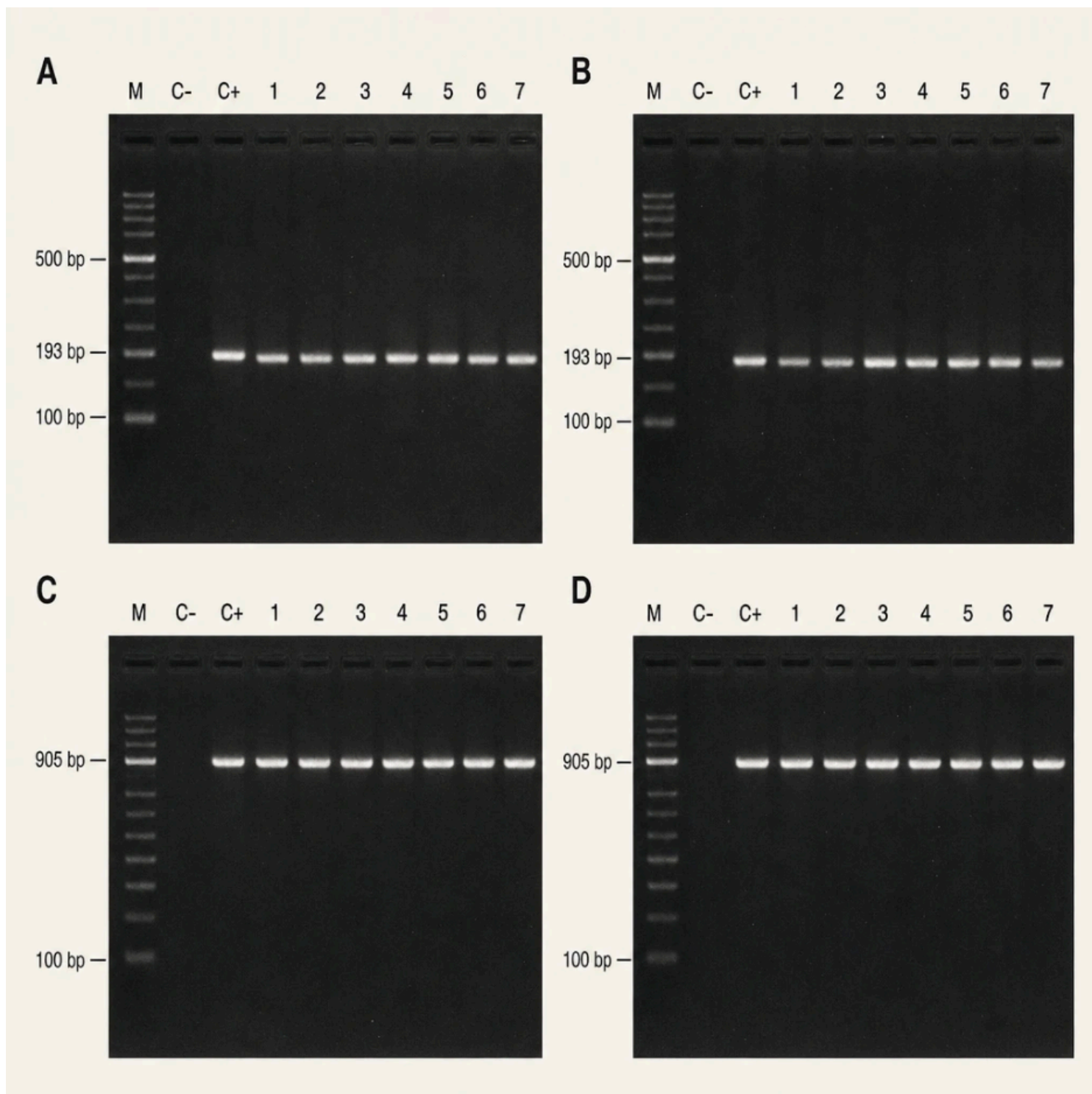


**Figure 3**

Molecular corroboration via PCR amplification of *Brucella* target genes resolved on 1.5% agarose gels. **(A)** Amplification of the *omp-2* gene (193 bp) from serial dilutions of extracted DNA in distilled water. **(B)** Corresponding *omp-2* amplification in a bovine serum matrix, illustrating reduced analytical sensitivity. **(C)** Amplification of the *16S rRNA* gene (905 bp) in distilled water, demonstrating superior detection limits. **(D)** Corresponding *16S rRNA* amplification in the serum matrix. (M: 100-bp DNA ladder; C-: Negative Control; C+: Positive Control; Lanes 1–7: serial 10-fold template dilutions).

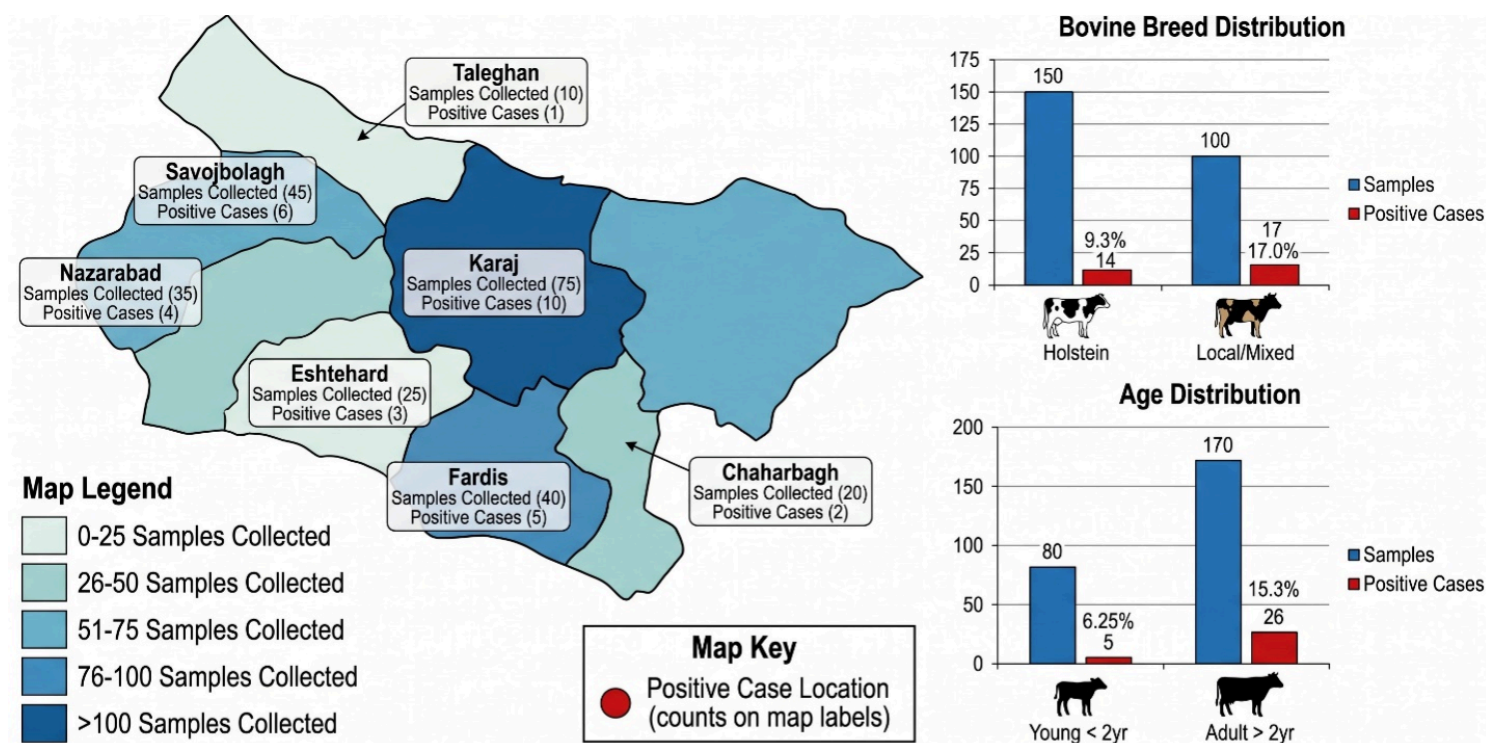


**Figure 4**

**Epidemiological and demographic distribution of bovine brucellosis cases in Alborz Province, Iran.** The geographical map (left) illustrates the spatial distribution of the 250 collected serum samples and the 31 confirmed positive cases across seven distinct counties. The bar charts (right) present the prevalence of infection stratified by bovine breed (Holstein vs. Local/Mixed) and age groups (<2 years vs. >2 years), highlighting higher positivity rates among adult and mixed-breed populations.