Development and Evaluation of an Enzyme-Linked Immunosorbent Assay (ELISA) for the Diagnosis of *Babesia ovis* Infection in Sheep

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Abstract

Background: Babesiosis causes significant economic losses in tropical regions like Iran. While microscopy misses subclinical carriers and PCR is costly for routine use, crude antigen ELISA offers a practical, affordable alternative for resource-limited laboratories.

Objectives: This study aimed to develop and optimize an indirect enzyme-linked immunosorbent assay (iELISA) for detection of *Babesia ovis* antibodies using crude antigens. We also monitored the temporal correlation between parasitemia and serological responses in an experimentally infected sheep.

Methods: Crude *B. ovis* antigen was prepared from infected sheep erythrocytes. The iELISA was optimized via checkerboard titration (antigen: 2 µg /mL; serum dilution: 1:100; HRP-conjugated anti-sheep IgG: 1:25,000). Diagnostic performance was evaluated using 20 positive and 32 confirmed negative sera. A healthy lamb was experimentally infected with cryopreserved *B. ovis*-infected blood. Monthly sampling included EDTA blood for PCR, serum for iELISA, and Giemsa-stained smears.

Results: The iELISA demonstrated 85% sensitivity, 84% specificity, 85% accuracy, 77% positive predictive value (PPV), and 90% negative predictive value (NPV). In the infected lamb, fever and acute parasitemia appeared on day 10 post-infection and resolved after imidocarb treatment. On day 12, microscopy, PCR, and iELISA were positive. During the carrier phase, microscopy was negative, while PCR and iELISA remained positive. By day 270, both microscopy and PCR were negative, but iELISA remained positive, indicating a persistent humoral immune response.

Conclusion: The developed crude antigen-based iELISA serves as a cost-effective tool for initial serological screening of *B. ovis* in sheep. With a high NPV (90.0%), it is particularly effective for screening, with PCR reserved for confirmation.

Keywords: *Babesia ovis,* ELISA, Soluble Piroplasm Antigen (SPA), Diagnosis, Sheep

1. Introduction

Ovine babesiosis, primarily caused by *Babesia ovis*, is a tick-borne disease of significant veterinary importance in tropical and subtropical regions. The disease is characterized by fever, anemia, and hemoglobinuria and, in severe cases, high mortality rates. In the absence of an effective vaccine, control strategies rely heavily on chemotherapy, tick control using acaricides, and proper pasture management [4].

In Iran, ovine babesiosis is a highly endemic and economically devastating disease, with *Babesia ovis* being the most prevalent species widely distributed across the country, particularly in the western, northwestern, and northeastern provinces where its primary tick vector (*Rhipicephalus bursa*) is abundant [4, 6]. Clinically, *B. ovis* is of paramount importance compared to other ovine piroplasms. While species such as *Babesia motasi* generally cause mild or even asymptomatic

infections in sheep, *B. ovis* is highly pathogenic. It induces severe clinical manifestations, including acute hyperthermia, severe hemolytic anemia, hemoglobinuria, and high mortality rates in susceptible flocks, thereby causing substantial economic losses in the Iranian sheep industry [8, 14].

The economic impact of babesiosis is profound, affecting meat and milk production, imposing treatment costs, and restricting livestock exports. Therefore, the establishment of a reliable diagnostic method is crucial for epidemiological monitoring and the implementation of control measures [7, 8, 14].

While microscopic examination of Giemsa-stained blood smears remains the gold standard for diagnosing acute cases, it has low sensitivity for detecting carrier animals with low parasitemia. Molecular methods, such as Polymerase Chain Reaction (PCR), offer high sensitivity and specificity but are often expensive and require specialized equipment, making them less suitable for large-scale screenings. Serological tests, particularly the Enzyme-Linked Immunosorbent Assay (ELISA) and Indirect Fluorescent Antibody Test (IFAT), are valuable for detecting past exposures and carrier states [1, 2, 13].

This study aims to establish an indirect ELISA (iELISA) for the detection of *B. ovis* antibodies in sheep, evaluate its performance using experimentally infected lambs monitored over a 9-month period, and compare antibody titers, PCR positivity, and traditional microscopic findings over time.

2. Materials and methods

2.1. iELISA development

2.1.1. Antigen preparation

An iELISA was established to detect antibodies against *B. ovis*. The Soluble Piroplasm Antigen (SPA) was prepared from *B. ovis*-infected erythrocytes following the method described by Waltisbuhl et al., [17] with minor modifications. Briefly, blood with high parasitemia was centrifuged, and the plasma and buffy coat were removed. The infected red blood cells (RBCs) were washed three times with cold Phosphate-Buffered Saline (PBS). The RBCs were then lysed using cold sterile distilled water at 4°C and subjected to repeated freeze-thaw cycles to release the intracellular piroplasms. Piroplasms were pelleted by centrifugation at 12,000 × g for 30 minutes at 4°C. The pellet was washed three times with PBS (suspension and centrifugation) and resuspended in one to two volumes of PBS. The crude soluble antigen were sonicated using a sonicator at medium power for 60–90 seconds. The resulting suspension was ultracentrifuged at 105,000 × g for 60 minutes at 4°C. The supernatant was collected and its protein concentration was determined using a NanoDrop spectrophotometer. Absorbance was measured at 280 nm against a blank (PBS buffer). The prepared SPA was stored at −70°C until use.

2.1.2. Checkerboard titration

Antigen and antibody concentrations were optimized using checkerboard titration [3], testing antigen concentrations from 0.5 μg/mL to 10 μg/mL and serum dilutions from 1:50 to 1:800. It is worth noting that the stock antigen was serially diluted to prepare various working concentrations based on the initial concentration measured by NanoDrop. Colorimetric detection was performed with an HRP-conjugated anti-sheep IgG and a TMB-based substrate solution.The optimal parameters — 2 μg/mL antigen and 1:100 serum dilution — were determined based on signal-to-noise (S/N) ratios (Mean OD positive/Mean OD negative).

2.1.3. *iELISA procedure and sera samples*

The indirect ELISA was performed following standard procedures based on the optimized parameters. Flat-bottom 96-well microtiter plates (Biosharp life Science, Beijing China) were coated with 100 μL/well of the prepared SPA diluted in carbonate-bicarbonate buffer (pH 9.6) at the optimal concentration of 2 μg/mL. The plates were then incubated overnight at 4°C. Following incubation, the plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Each wash lasted for 5 minutes, with 300 μL of wash buffer added to each well.

To prevent non-specific binding, the wells were blocked with 200 μL of blocking buffer (5% skim milk in PBST) and incubated for 1 h at 37°C. After washing three times with PBST, 100 μL of serum samples (diluted 1:100 in blocking buffer) were added to duplicate wells and incubated for 1 h at 37°C.

Following another triple washing step with PBST, 100 μL of horseradish peroxidase (HRP)-conjugated anti-sheep IgG (diluted according to the manufacturer's instructions; 1: 25000) was added to each well and incubated for 1 h at 37°C. The plates were washed five times with PBST to remove any unbound conjugate. For color development, 100 μL of TMB was added to each well and incubated for 15 min at room temperature in the dark. The enzymatic reaction was stopped by adding 50 μL/well of stopping solution (1 M H_2SO_4), and the optical density (OD) was immediately measured at 450 nm using a microplate reader.

To validate the ELISA, its diagnostic performance was evaluated using three well-characterized serum panels:

Positive panel (n=20): Sera sourced from sheep with natural *B. ovis* infections, where each case was confirmed by both microscopic examination of blood smears and a species-specific PCR. Negative panel (n=23): This panel comprised sera from two distinct, verified *B. ovis*-free sources: (a) twenty-three sera from a certified parasite-free flock maintained at Razi Vaccine and Serum Research Institute (RVSRI) for vaccine production, and (b) nine sera from clinically healthy lambs in tick-free regions with no history of babesiosis. Specificity panel (n=9): To assess potential cross-reactivity, this panel included sera containing antibodies against other common ovine pathogens: *Toxoplasma gondii*, *Neospora caninum*, and *Theileria lestoquardi*. To rigorously exclude subclinical infections, all 32 negative sera were confirmed for *B. ovis* DNA using a highly sensitive PCR assay.

Genomic DNA was extracted from EDTA-anticoagulated blood samples of both positive and negative and amplified by PCR targeting a specific region of the *B. ovis* ATP-binding protein gene, as described by Habibi et al. [6]. In this study, PCR served as the definitive molecular method to detect active infections, identify asymptomatic carrier animals, and validate the serological findings.

To ensure the reliability of the amplifications, strictly validated controls were included in each PCR run. Genomic DNA extracted from the blood of a confirmed *B. ovis*-infected sheep served as the positive control. The negative controls comprised DNA extracted from a healthy, uninfected sheep, alongside a no-template control (nuclease-free water), which were included in every amplification cycle.

It is worth noting that this specific PCR assay, in conjunction with microscopic examination, was utilized to rigorously confirm the true-negative status for the 9 heterologous parasite sera (*Theileria lestoquardi*, *Toxoplasma gondii*, and *Neospora caninum*) prior to their use in the ELISA cross-reactivity evaluation.

2.1.4. iELISA Cut-off determination and performance

The diagnostic cut-off value for the ELISA was established using a panel of 23 confirmed-negative sheep sera. The threshold was defined as the mean OD of these negative samples plus three standard deviations (Cut-off = Mean OD + 3SD). Samples yielding an OD value equal to or greater than this threshold were considered seropositive. To assess the sensitivity and specificity (including the potential for cross-reactivity), the assay was further evaluated using a panel of 20 positive sera and 32 negative sera.

For the evaluation of the ELISA's diagnostic performance, a combined approach utilizing microscopic examination and specific PCR was designated as the composite "gold standard" reference. True positive and true negative statuses of the field and reference sera were defined based on the concordant results of these two methods.

2.2. Experimental *B. ovis* infection model

This preliminary study was conducted between 2023 and 2025 at the Department of Parasitic Vaccine Research and Production, RVSRI, Karaj, Iran. To establish baseline ELISA performance, a single healthy male lamb (>8 months old) was experimentally infected as a controlled infection model, alongside validation using archived positive/negative sera panels.

The lamb was housed in tick-free, acaricide-treated facilities following a 2-week quarantine with daily health monitoring (rectal temperature, smears, PCR confirming *Babesia*-negative status using genus/species-specific primers). A cryopreserved *B. ovis* isolate (RVSRI-maintained) was reactivated in splenectomized lambs; 15 ml of peak-parasitemia blood was intravenously inoculated into the experimental lamb, followed by daily monitoring for hyperthermia and parasitemia.

Monthly blood samples were collected for nine months post-infection: EDTA tubes for PCR (DNA extraction) and plain tubes for serum (ELISA; centrifuged, aliquoted, and stored at −20°C). Ear-vein thin smears enabled microscopy. Sera from this longitudinal experimental infection were 12 time points. Peripheral thin smears were air-dried, fixed with absolute methanol, and stained with 10% Giemsa. Each slide was examined under an oil-immersion lens (1000× magnification) across at least 50 fields to detect intra-erythrocytic piroplasms. PCR and iELISA were conducted on samples collected at each time point, following the methods described above.

2.3. Statistical Analysis

The diagnostic indices, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, false positive rate (FPR), false negative rate (FNR), and likelihood ratios (LR+ and LR−), were calculated. The data were analyzed using GraphPad Prism version 8 for Windows (https://www.graphpad.com/quickcalcs/kappa1/) and the online tool MedCalc (https://www.medcalc.org/en/calc/diagnostic_test.php). The diagnostic performance of ELISA in terms of sensitivity, specificity, and predictive values was evaluated statistically using the MedCalc diagnostic test assessment tool (https://www.medcalc.org/en/calc/diagnostic_test.php).

Given the longitudinal design involving a single experimentally infected lamb, inferential statistical comparisons were not applicable. Instead, temporal trends in parasitemia, antibody titers, and PCR positivity were described using descriptive statistics and visualized through line graphs to illustrate the infection dynamics over the nine-month monitoring period.

3. Results

3.1. Establishment of iELISA

The iELISA based on crude soluble antigen of *Babesia ovis* was successfully standardized using 2 µg/mL antigen concentration and a serum dilution of 1:100 in PBS-T. The optimal diagnostic cut off value was determined as 0.3157.

Validation using 20 confirmed positive and 32 negative sera yielded 17 true positives, 3 false negatives, 5 false positives, and 27 true negatives. The assay demonstrated a sensitivity of 85.0% (95% CI: 62.1–96.8%), specificity of 84.4% (95% CI: 67.2–94.7%), positive predictive value of 77.3%, and negative predictive value of 90.0%. The overall diagnostic accuracy was 84.6% (95% CI: 71.92%- 93.12%), with likelihood ratios of $LR^{+} = 5.44$ and $LR^{-} = 0.178$.

3.2. Clinical observations in experimentally animal

Following experimental inoculation, the lamb developed clinical manifestations of ovine babesiosis. A gradual rise in rectal temperature was recorded beginning on day 10 post-inoculation, coinciding with the onset of the acute phase.

Microscopic examination of peripheral blood smears confirmed the presence of parasitemia during the febrile period. After antiprotozoal treatment, parasitemia markedly decreased and subsequently disappeared in later examinations, consistent with recovery and clearance of *Babesia ovis* from circulation.

3.3. Comparison of diagnostic methods in experimentally infected lamb

Microscopic examination detected *Babesia ovis* only during the acute febrile phase, coinciding with high parasitemia levels. No parasites were seen during chronic or carrier stages in monthly follow ups. After the onset of clinical signs, imidocarb dipropionate treatment was administered; within ten days, only trace parasitemia remained, confirming recovery (Figure 1).

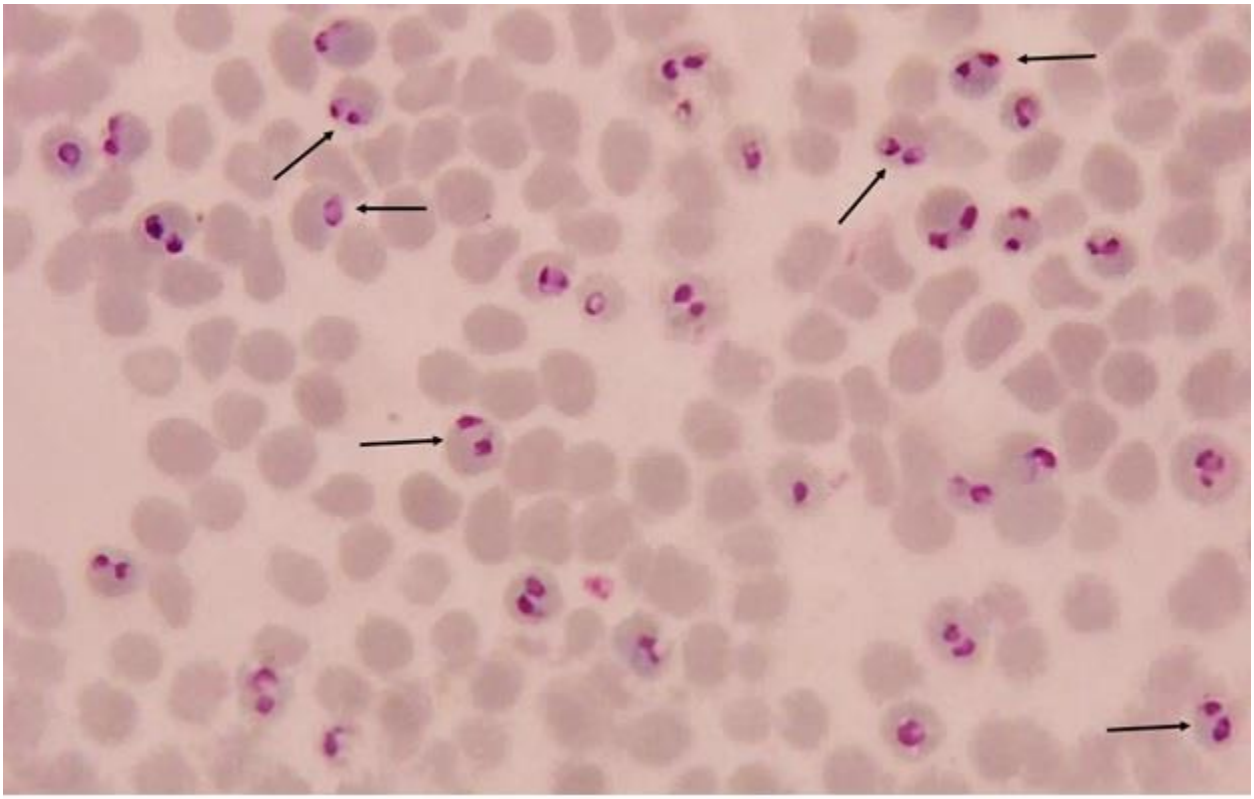

Figure 1. Piroplasm forms of ***Babesia ovis*** observed within the erythrocytes of a lamb experimentally infected with ovine babesiosis. The intraerythrocytic parasites appear as pear-shaped, frequently paired piroplasms (indicated by arrows). Magnification: ×1000.

The PCR assay exhibited superior sensitivity, detecting *B. ovis* DNA even when blood smears were negative and remaining positive until approximately day 200, confirming prolonged parasite DNA persistence beyond clinical recovery.

The iELISA (cut off = 0.3157) successfully detected antibodies from the acute stage onward and maintained seropositivity for over 250 days in experimentally infected animal.

The assay showed high sensitivity for detecting antibodies in experimentally infected animal, becoming positive from approximately three weeks post infection.

Antibody titers remained detectable throughout the nine month monitoring period, including stages when parasitemia was no longer visible on blood smears. This persistence indicates a sustained humoral immune response and long-term immunological memory against *B. ovis*, with a stabilization phase observed between days 100–250 post infection (Figure 2).

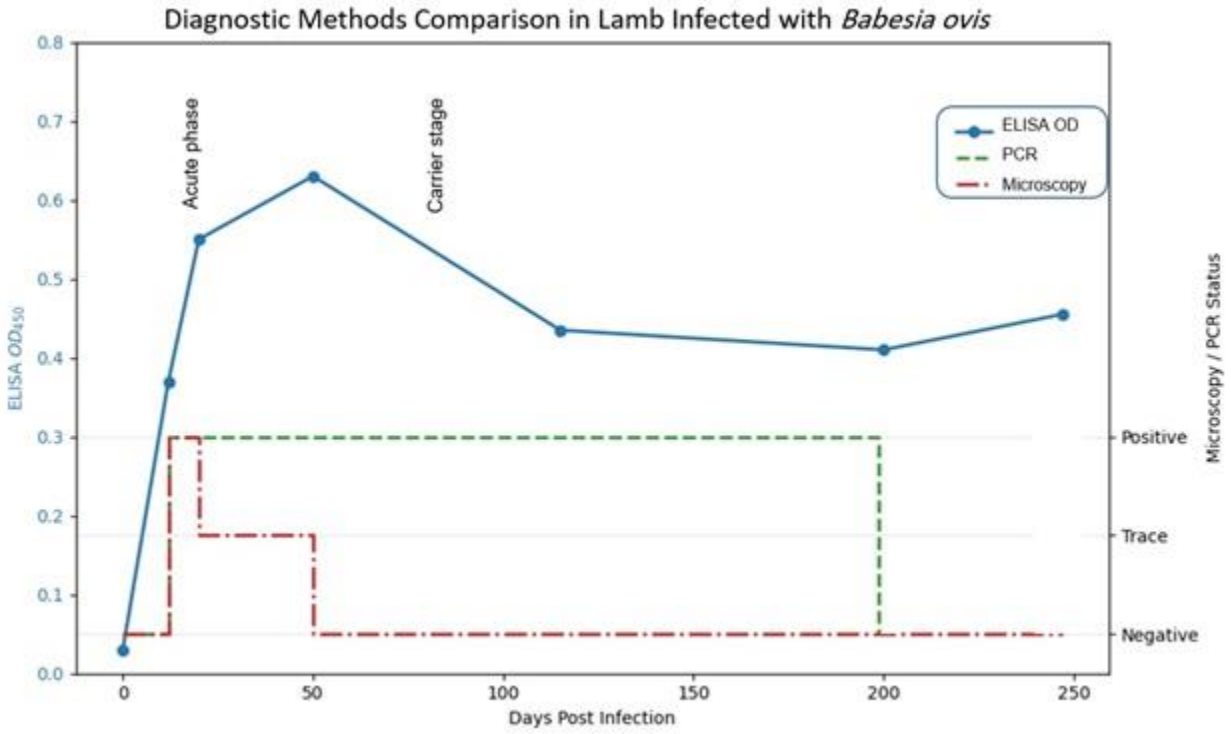

Figure 2. Comparative assessment of ELISA, PCR, and microscopy in a lamb experimentally infected with *Babesia ovis* over time. ELISA OD_{450} values indicate the progression of the humoral response, peaking during the acute phase and gradually declining through the carrier stage. PCR remained positive from early infection to day 200, reflecting persistent parasitemia, while microscopy showed positivity only during the acute stage. Overall, the figure illustrates the temporal diagnostic dynamics of the three methods and the superior sensitivity of PCR in chronic and low-parasitemia phases.

The nearly parallel persistence of PCR positivity and sustained antibody titers (until day 200) suggests that the lamb may have entered a carrier state, reflecting the continuous presence of parasite DNA alongside a long-term humoral immune response. For microscopic examinations,

the observation of 1-2 infected red blood cells (iRBCs) per field of view was recorded as "Trace", while counts exceeding 5-10 iRBCs per field were considered "High" (Table 1).

Table 1. Time-based comparison of diagnostic methods in the experimentally infected lamb with *Babesia ovis*

Day post-infection	Clinical status	Microscopy	PCR	ELISA OD_{450}	Interpretation
0 (Pre-infection)	Normal, baseline	Negative	Negative	0.03	Uninfected control
12 (acute onset)	Fever, Anemia, Parasitemia	Positive (high)	Positive	0.37	Acute phase — active infection
20 (after treatment)	Recovery phase	Trace	Positive	0.55	Declining parasitemia; antibody rising
50	Afebrile, clinically normal	Negative	Positive	0.63	Post-treatment carrier stage
70 – 160	Stable, Normal, No symptoms	Negative	Positive	0.47–0.40	Seroconversion plateau; sustained humoral response, persistent DNA, low-level carrier
200	Normal	Negative	Positive	0.41	Antibody persists
230–265	Normal	Negative	Negative	0.45–0.46	PCR turns negative; Chronic phase stabilization; immune memory

4. Discussion

The accurate diagnosis of *Babesia ovis* infection remains a basis for effective control and epidemiological surveillance of ovine babesiosis, particularly in endemic regions where subclinical and chronic infections compromise herd health and productivity. Although *B. ovis* infection is endemic in Iran [6-9, 14], the reliability of epidemiological data has historically been constrained by heterogeneous diagnostic practices and limited access to standardized serological tools.

The present study addresses this gap by evaluating a locally developed iELISA based on SPA of *B. ovis*, offering a practical, regionally adaptable diagnostic platform for large-scale screening. As a preliminary evaluation, the present study used a limited panel of confirmed positive (n=20) and negative (n=32) sera available from prior collections to establish baseline performance metrics.

Microscopic examination of blood smears, while effective during acute infection phases characterized by high parasitemia, proved inadequate for detecting low-level parasitemia in chronic or carrier animals during monthly follow-up. This diagnostic limitation is well-documented in endemic settings and supports the hypothesis that subclinical carriers serve as reservoirs maintaining transmission via tick vectors in the absence of overt clinical signs [4]. Consequently, serological assays such as ELISA have emerged as essential tools for herd-level

surveillance, particularly when combined with molecular methods such as PCR for confirmation of active infections.

Performance assessment of the developed SPA-based iELISA revealed a sensitivity of 85.0%, a specificity of 84.4%, and a promising overall accuracy of 84.6%, making it comparable to existing serological methods reported in the literature [1, 2]. While the assay exhibited a notably high NPV of 90.0%, confirming excellent reliability for ruling out infection at the herd level, the PPV of 77.3% warrants careful consideration. This PPV indicates that approximately one in four positive results yielded by the test may be a false positive. In veterinary practice, this carries significant economic and clinical implications, including unnecessary treatment or culling of animals, as well as the necessity for supplementary confirmatory tests which can add to the costs. Therefore, while the sensitivity and specificity of the assay are at acceptable levels for crude-antigen platforms, its moderate PPV necessitates caution when interpreting positive results. These analytical findings strongly suggest that the assay is most effectively utilized as a primary screening or adjunctive diagnostic tool rather than a definitive standalone test.

These performance metrics, while sufficient for resource-limited settings, are notably lower than those reported for ELISAs employing purified recombinant antigens. Studies utilizing recombinant proteins such as *B. ovis* secreted antigen-1 (rBoSA1) and apical membrane antigen-1 (rBoAMA1) have routinely demonstrated specificities ≥90–95% with minimal or no cross-reactivity against phylogenetically related parasites [1, 15, 16]. For instance, Sevinc et al. [15] first characterized rBoSA1, demonstrating antibody detection as early as 7–8 days post-infection using an iELISA format. Subsequent work by Sevinc et al. [16] advanced this approach with a sandwich ELISA capable of detecting native BoSA1 protein in blood during very early infection stages, even before microscopically detectable parasitemia. Similarly, Ozubek et al. [12] introduced spherical body protein 4 (SBP4) as a novel recombinant antigen, showing strong immunoreactivity with sera from naturally infected sheep and no cross-reactivity with negative controls.

Although positive results increase the likelihood of exposure (LR+ = 5.44), they may still reflect past infection or cross-reactivity *(*i.e., *Theileria lestoquardi*, *Toxoplasma gondii*, and *Neospora caninum*), as indicated by the presence of five false-positive and three false-negative samples. Therefore, while ELISA is a useful tool for herd-level surveillance and preliminary diagnosis, confirmatory testing, especially PCR, remains essential for identifying active infections.

Regarding the cross-reactivity, antigenic similarity is a recognized limitation of crude-antigen ELISAs and accounts for the observed false-positive results. Nevertheless, evaluation with heterologous positive sera confirmed that the assay retained satisfactory diagnostic specificity for practical field use. In regions where multiple hemoparasites co-circulate, such as northwestern Iran [10] and northeast Brazil [11], positive ELISA results should be interpreted cautiously and confirmed by molecular assays to prevent misclassification.

In experimentally infected animal, the kinetics of the ELISA response mirrored the typical pattern of humoral immunity: OD values rose rapidly post-infection, peaked between days 30 and 60, and remained stable up to day 270, even after parasitemia clearance around day 200 as confirmed by PCR. This persistence of antibodies supports the concept of long-term seropositivity and highlights

the potential of iELISA for monitoring herd-level exposure and immune response rather than acute infection. This finding is consistent with the observations of Firat et al. [5], who employed ELISA alongside microscopy and PCR to monitor chronic *B. ovis* infections, confirming its value for long-term infection surveillance even in clinically recovered animals.

ELISA and PCR were demonstrated to be complementary methods: PCR provides higher sensitivity for detecting active infections and carriers, while ELISA offers significant advantages for large-scale screening due to its throughput, simplicity, and cost-effectiveness. When interpreted within the framework established by recombinant antigen-based studies [12, 15, 16, 18], the current SPA-based ELISA represents a practical, cost-effective, and regionally adaptable diagnostic tool. Unlike recombinant antigen platforms, which provide high specificity and batch-to-batch consistency but require advanced molecular infrastructure, the SPA-based ELISA relies on locally obtainable parasite material and presents a broad spectrum of native epitopes, potentially enhancing sensitivity during polyclonal immune responses under heterogeneous field conditions.

A primary limitation of this study is the use of a single experimentally infected lamb for the *in vivo* validation phase. This single-animal model, while effective for demonstrating the proof-of-concept of seroconversion and tracking the initial temporal dynamics of the antibody response, inherently lacks biological replicates. This design prevents any statistical analysis of individual variation in immune responses and limits the generalizability of our kinetic findings. The primary performance characteristics of the assay (e.g., sensitivity and specificity) were determined using a larger, controlled panel of archived sera (n=52). However, to confirm the consistency and reproducibility of the immune response timeline observed here, future longitudinal studies involving a statistically significant cohort of animals are essential. Such studies would allow for a more robust evaluation of the iELISA's performance in capturing the full spectrum of host-pathogen interactions at the population level.

The moderate specificity observed in this study highlights the importance of transitioning to recombinant antigens (such as rBoSA1, rBoAMA1, or SBP4) in future versions of the assay. Incorporating these refined antigens is expected to minimize non-specific reactions, improve the PPV, and enhance the overall diagnostic reliability for both epidemiological surveillance and clinical applications in endemic regions of Iran. Nevertheless, the current assay's robustness, accessibility, and high NPV support its use as a practical first-line screening tool, provided that ELISA-positive results are confirmed by molecular methods. This dual-step diagnostic approach—combining ELISA for initial screening and PCR for confirmation—ensures accurate discrimination between mere exposure and active infection, thereby strengthening disease control and epidemiological monitoring programs in resource-limited endemic settings.

Conclusion

In conclusion, the SPA-based iELISA evaluated in this study provides a practical, cost-effective, and regionally adaptable tool for the serological screening of *Babesia ovis* infection in sheep. Demonstrating an acceptable overall accuracy of 84.6% and a particularly high negative predictive value, the assay effectively supports ruling out infection at the herd level. However, given its

moderate positive predictive value (77.3%) and the inherent risk of false-positive results associated with crude antigen-based assays, its implementation as a standalone tool in broad national screening programs must be approached with caution at this stage. Currently, the assay shows considerable potential as an adjunctive diagnostic tool and is best suited for targeted high-risk populations or as a first-tier screening test, where positive findings strictly require confirmation via gold-standard molecular methods. Future research and larger-scale cohort studies are essential to further optimize the assay's performance and fully determine its clinical utility for epidemiological monitoring and control programs in endemic areas.

Authors' Contribution

Conceptualization: G.H., G.K.; **Methodology:** G.H., S.F.; **Formal analysis:** S.F.; **Investigation (Laboratory and Field operations):** A.A., S.F., A.S.; **Resources (Sampling):** A.A., A.S.; **Writing – original draft preparation:** G.H.; **Writing – review & editing:** G.H., S.F.; **Supervision and Project administration:** G.K. All authors read and approved the final manuscript.

Ethics Approval

In vivo procedures, including animal inoculations for *Babesia* propagation, were conducted following routine Standard Operating Procedures (SOPs) in the Parasitic Vaccine Production Department at the Razi Vaccine and Serum Research Institute. These methods have been historically established for *in vivo* multiplication of the protozoan and adhered to institutional animal welfare guidelines. Due to the routine nature of these protocols, separate ethical approval for this study was not sought. This study continues the previously concluded national projects (Project # 2-024-250000-21-83008 and 0-18-18-023-960370), which validated these standard procedures.

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Conflict of interest

The authors declare that they have no conflicts of interest related to the content of this manuscript.

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Data availability

The datasets generated and analyzed during the development and validation of the ELISA assay, including the raw OD values and the calculated sensitivity and specificity metrics, are available from the corresponding author upon reasonable request.

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