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# Serological Investigation of Antibodies Induced by Smooth and Rough *Brucella* Lipopolysaccharide in Sheep

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## ABSTRACT

Different *Brucella* species, characterized by smooth and rough cell walls, are recognized as etiological agents of ovine brucellosis. While serological studies in adult and older sheep are common, those on the serological status of young animals are limited. The present study aimed to investigate brucellosis in sheep caused by smooth- and rough-walled *Brucella* species at two slaughterhouses in the Marmara Region of Turkey using serological methods. Three different analyses, including the rapid slide test, the tube agglutination test, and the indirect enzyme-linked immunosorbent assay (iELISA), each included smooth- and rough-structured *Brucella* antigens, resulting in a total of six separate tests. Blood sera were randomly obtained from 150 male sheep, approximately 1 year old and weighing about 70 kilograms, from the Merino, Kıvırcık, Lacauné, and Tahirova breeds. These sheep were brought to slaughterhouses from several provinces, including 25 from Tekirdağ, 45 from İstanbul, 43 from Kırklareli, 17 from Çanakkale, and 20 from Kocaeli. Using the rough iELISA, 15 positive cases (10%) were identified, of which eight were concurrently positive with the smooth iELISA. The low positivity rate and negative results from tube agglutination and rapid slide tests decreased the probability that the sheep had active brucellosis. Positive results for the same sample in both S-iELISA and R-iELISA tests may be linked to smooth LPSs, while positivity only in R-iELISA could be attributed to rough LPSs. Although no *Brucella ovis* or *Brucella melitensis* were found in the animals examined in the present study, the positive ELISA results suggested that brucellosis could have important epidemiological implications. Using two iELISA methods with smooth and rough LPS antigens concurrently enhanced the diagnostic sensitivity of indirect serological diagnosis of brucellosis.

**Keywords:** Brucellosis, Sheep, Slaughterhouse, Rough lipopolysaccharide, Smooth lipopolysaccharide

## INTRODUCTION

Brucellosis is a general term referring to the disease caused by bacteria of the genus *Brucella* (*B.*). These *Brucella* species exhibit host specificity; nonetheless, they may infect animals other than their specific hosts. Sheep serve as specific hosts for both *B. melitensis* and *B. ovis* (Alton et al., 1988; WOAH, 2025a). *Brucella melitensis* is the most common species in sheep and the predominant agent of ovine brucellosis (WOAH, 2025a). *Brucella melitensis* infections have long been recognized and remain widespread in Turkey. In Turkey, different control strategies have been implemented to manage brucellosis, specifically *B. melitensis* infections, since the 1980s (Çakır and Yıldırım, 2018). According to the latest national studies, the individual prevalence of ovine and caprine brucellosis in Turkey was estimated at 2.10%, while herd prevalence was 12.34% (Bebek, 2019). In similar epidemiological studies, animals under one year of age were typically excluded from risk groups for statistical reasons. This exclusion was due to the higher likelihood of false positives in young animals, which could be attributable to maternal antibodies or vaccination. While this approach might be epidemiologically justified, it can limit the serological assessment of young animals. Brucellosis is frequently correlated with abortions and reproductive disorders in female animals. In males, infection leads to considerable productivity and economic losses, including epididymitis, orchitis, testicular atrophy, reduced sperm quality, and infertility. The presence of testicular abnormalities in male animals serves as a significant diagnostic indicator of brucellosis. In young animals, subclinical or latent infections may occur, thereby facilitating the persistence and transmission of brucellosis within herds (WOAH, 2025a;b). Monitoring young male animals for brucellosis can provide valuable epidemiological data for herd management.

Numerous studies have addressed the diagnosis of *B. ovis* infections in sheep (Praud et al., 2012; Costa et al., 2016; Branscom et al., 2018). *Brucella ovis* has been reported in multiple countries, and in regions where disease prevalence



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posed a significant threat, effective control strategies have been implemented (Galluzzo et al., 2021; WOAH, 2025b). In Turkey, there is no dedicated control program for managing *B. ovis* infections in sheep (Keskin et al., 2022). Apart from a single bacterial isolation from a ram imported from Syria (Erdenliğ Gürbilek et al., 2018), there have been no comprehensive studies on the prevalence or incidence of *B. ovis* infections across all provinces in Turkey. However, numerous serological studies have indicated *B. ovis* seropositivity (Uçan and Aras, 2007; Tel et al., 2016; Keskin et al., 2022), indicating a strong likelihood of *B. ovis* presence in Turkey.

*Brucella* are classified as smooth or rough strains based on their Gram-negative cell wall structure. The lipopolysaccharide (LPS) component of the cell wall is the primary determinant of this classification. Smooth *Brucella* strains have an S-LPS molecule on their surface, in which the O-polysaccharide (O-PS) is a key determinant of the cell wall structure. Naturally rough strains, including *B. ovis*, *B. canis*, *B. abortus* RB51, and *B. melitensis* B115, do not include this O-PS molecule. Thus, the presence or absence of O-PS distinguishes smooth strains from rough strains (Nielsen et al., 2004). Field isolates from diseased animals typically are smooth *Brucella* strains, although there have been occasional reports of stable rough field strains (Yang et al., 2020). Some rough strains originate from smooth ones that are compelled to grow under unfavorable conditions, such as stagnant liquid media. Consequently, isolation and preservation conditions significantly influence the bacterial cell wall structure of these isolates (Alton et al., 1988).

The gold-standard test for diagnosing brucellosis is bacterial isolation and identification, although these methods are expensive and time-consuming (Saytekin and Ak, 2018). Additionally, bacterial isolation carries a high risk of contamination, endangering personnel and the environment. Therefore, it is essential to carefully assess and establish the laboratory biosecurity level before handling *Brucella* bacteria (WOAH, 2025a). Many molecular diagnostic methods require direct DNA extraction from bacteria, increasing the risk of bacterial contamination throughout the process. These methods are beneficial because they provide direct diagnosis for brucellosis (Saytekin and Ak, 2018). In contrast to bacterial isolations, serological analyses pose a lower risk of contamination and are more cost-effective and less labor-intensive. However, indirect serological diagnosis has lower specificity because cross-reactions can occur among closely related bacterial species. Therefore, selecting appropriate antigens for serological analyses is important (WOAH, 2025 a;b). Furthermore, Alton et al. (1988) noted that structural differences among *Brucella* strains necessitate selecting suitable serological test antigens, either smooth or rough, depending on the specific strain targeted. Advancements in technology have led to more precise and sensitive serological diagnostic tests. The World Organization for Animal Health (WOAH) noted different assays and tests for diagnosing infections caused by the smooth (*B. melitensis*) and rough (*B. ovis*) *Brucella* species (WOAH, 2025a;b). These methods included several types of enzyme-linked immunosorbent assay (ELISA), a highly sensitive and reliable analytical method for detecting brucellosis, with variants depending on the target antigen and diagnostic goal (Keskin et al., 2022). The present study aimed to assess the presence of antibodies targeting both smooth and rough LPS phenotypes of *Brucella* species using several serological techniques.

## MATERIALS AND METHODS

### Ethical approval

This study received approval from the İstanbul University-Cerrahpaşa Faculty of Veterinary Science Research Ethics Committee under approval number 2021-44, dated October 26, 2021.

### Samples collection and location

Sample size calculation was conducted with a 95% confidence level, an 8% margin of error, and an anticipated prevalence of 50%. Between March 2022 and February 2023, a total of 150 serum samples were randomly collected from male Merino, Tahirova, Lacauné, and Kıvırcık sheep aged between 6 months and 1 year, with an average weight of 70 kg. These sheep were brought to two slaughterhouses located in Istanbul from several provinces, including 25 from Tekirdağ, 45 from İstanbul, 43 from Kırklareli, 17 from Çanakkale, and 20 from Kocaeli, Turkey (Figure 1). During the study period, slaughterhouses were visited every two months, and blood serum was collected from 15 animals during each visit.

The number of animals sampled from each province, their breeds, and the proportional distribution of the samples are presented in Table 1. All blood samples were drawn from the jugular vein using sterile, non-anticoagulant vacuum tubes in accordance with aseptic and antiseptic procedures. Following an overnight stand period, the samples were centrifuged to separate the sera. The collected sera were then transferred into sterile Eppendorf tubes, appropriately numbered, and stored at 2-8°C until further testing. All processes were carried out in the Microbiology laboratory of the Veterinary Faculty of İstanbul University-Cerrahpaşa, Turkey.

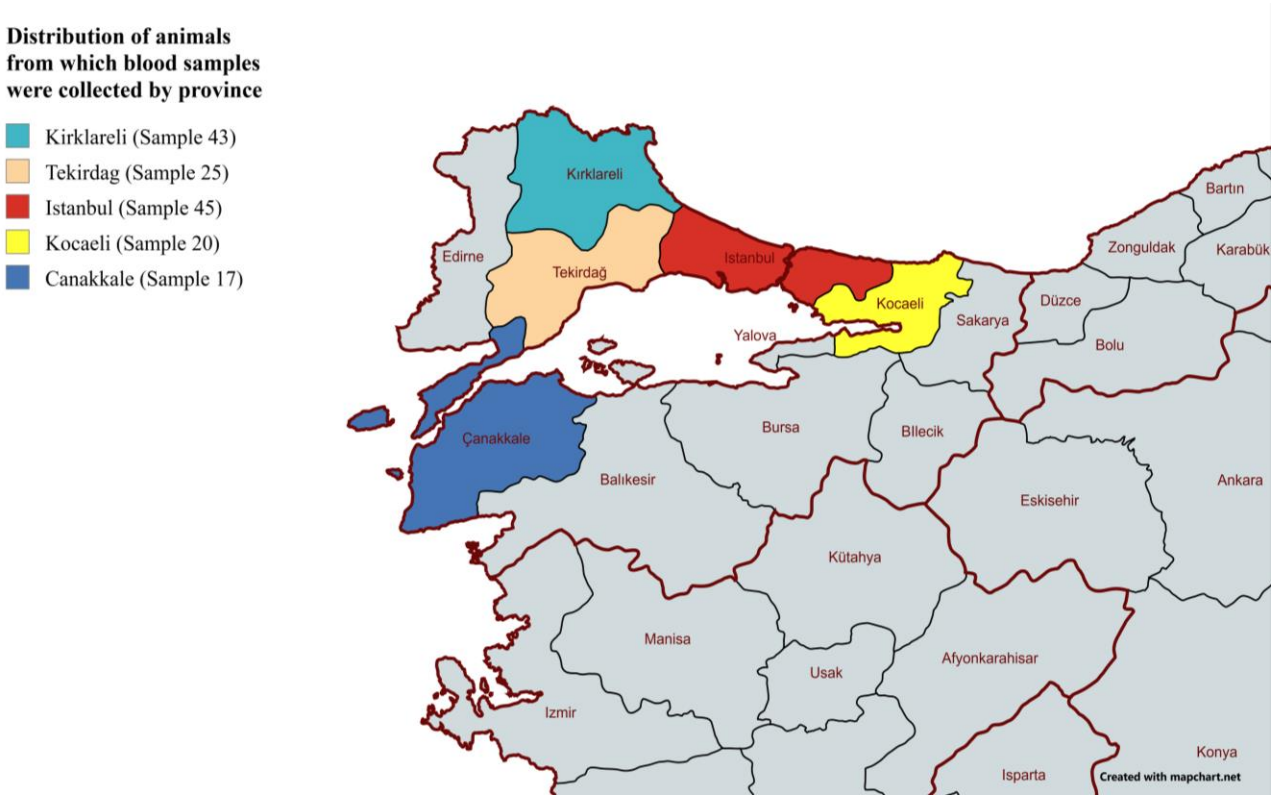


**Figure 1.** Distribution of sheep blood samples by province in Turkey. Source: Mapchart.net

**Table 1.** Distribution of sheep blood samples by breed and province, collected from slaughterhouses in Turkey between March 2022 and February 2023

| Province | Numbers of serum samples | Sheep breeds and numbers | Percentage |
|---|---|---|---|
| Çanakkale | 17 | Kıvırcık (17) | 11.33 |
| İstanbul | 45 | Kıvırcık Cross (32)<br>Merino (4)<br>Merino Cross (1)<br>Lacaune (5)<br>Tahirova (3) | 30 |
| Kocaeli | 20 | Merino Cross (20) | 13.33 |
| Kırklareli | 43 | Kıvırcık (30)<br>Kıvırcık Cross (13) | 28.67 |
| Tekirdağ | 25 | Merino (7)<br>Merino Cross (18) | 16.67 |
| Total | 150 | | 100 |

**Clinical examination**

The medical histories and general physical examinations of 150 animals were carried out in the slaughterhouses. Animal owners were asked if the source flocks of these sheep had a history of Brucellosis diagnosis, if any abortion cases had been reported, and whether any animals demonstrated signs of lameness and orchitis. General physical examinations of animals were performed, and body temperatures were measured. The presence of inflammatory signs, such as color change, heat, pain, and swelling in joints or the testis, was evaluated visually and by palpation.

**Serological analysis**

A total of 150 serum samples were examined for the serological diagnosis of Brucella species with either smooth or rough cell wall structures, utilizing six different tests. The initial analyses involved rapid slide agglutination tests prepared with antigens derived from standard *Brucella* strains possessing smooth and rough cell wall structures, specifically the S-Rose Bengal plate test (S-RBPT) and R-Rose Bengal plate test (R-RBPT). Subsequently, tube agglutination tests were performed; the S-tube agglutination test (S-TAT) and the R-modified 2-mercaptoethanol tube

agglutination test (R-2ME-TAT). Finally, indirect ELISA (iELISA) was performed to detect smooth (S-iELISA) and rough (R-iELISA) *Brucella* strains (Alton et al., 1988; WOAH, 2025a). Among these methods, S-RBPT and S-TAT were commercially acquired. The remaining assays were prepared in-house, adhering to established protocols documented in the studies.

**Reference strains and sera**

Reference strains and control sera were obtained from two sources. The *B. canis* M [-] strain and positive and negative control sera for the S-RBPT, R-RBPT, S-TAT, and R-2ME-TAT assays were provided by the *Brucella* reference diagnostic laboratory at the Pendik Veterinary Control Institute Directorate, Turkey. Additionally, the Pendik Veterinary Control Institute Directorate, Turkey, supplied the commercial S-RBPT and S-TAT kits (lot numbers RBPT/02/2022 and TAT/02/2022), prepared using the smooth *B. abortus* S99 strain. The R-RBPT and R-2ME-TAT kits were prepared in-house using the *B. canis* M [-] strain, following the method of Alton et al. (1988). Positive and negative control sera for the S-iELISA and R-iELISA with *B. melitensis* B115 were sourced from the Laboratories of the Department of Microbiology, Faculty of Veterinary Medicine, Harran University, Şanlıurfa, Turkey. Additional positive control sera for the R-iELISA were sourced from rams previously diagnosed with *B. ovis* (Erdenliğ Gürbilek et al., 2018). Both the S-RBPT and R-RBPT employed stained antigens and were performed by mixing 50 µL of serum with the antigen on a clean glass slide, with agglutination evaluated within four minutes (Alton et al., 1988; WOAH, 2025a).

The tube agglutination tests, S-TAT and R-2ME-TAT, which were prepared with unstained antigens, were performed in glass tubes. Ten serial dilutions of each suspected serum were prepared in clean glass tubes for each test. Equal volumes (0.5 mL) of the respective test antigen were then added, and after incubation at 37°C for 24 hours, the results were evaluated (Alton et al., 1988; WOAH, 2025a).

For the S-iELISA, ready-to-use S-LPS with a smooth structure was obtained from the United Kingdom Animal and Plant Health Agency (APHA) and used to coat microplates (NUNC, 269620, Denmark). For the R-iELISA, the rough-form antigen was prepared from the *B. melitensis* B115 standard strain by isolating the R-LPS layer using a modified Tri-Reagent method, as described by Yi and Hackett (2000). Colonies of *B. melitensis* B115 grown on tryptic soy agar (Merck, Germany) under microaerobic conditions were collected from the agar surface using sterile phosphate-buffered saline (PBS), inactivated by heating in a water bath at 80°C for 30 minutes. The resulting killed bacterial suspension was subsequently centrifuged at 3500 g at 4°C to remove the supernatant (Yi and Hackett, 2000). The sediment of the killed bacterial pellet was collected. For each gram of bacterial pellet, 2 mL of Tri-Reagent was added. The mixture was incubated at room temperature for 10-15 minutes to ensure complete homogenization. Subsequently, 300 µL of chloroform per gram of bacterial pellet was added to induce phase separation. The suspension was vortexed, incubated for an additional 10 minutes, and centrifuged at 12000 g for 10 minutes to separate the supernatant from the organic phase. The supernatant was transferred to a new 1.5 mL centrifuge tube. In the organic phase, 100 µL of distilled water was added, followed by centrifugation at 12000 g for 10 minutes. The resulting supernatant was combined with the previous aqueous extract, and the crude LPS solution was stored at –20°C for subsequent use as the solid-phase antigen in ELISA (Yi and Hackett, 2000).

Before conducting these analyses, different concentrations of the smooth and rough antigens were prepared and tested through reciprocal titrations (checkerboard analysis) to identify the optimal antigen concentration. Antigen was diluted in carbonate-bicarbonate coating buffer (pH 9.6) to the specified concentration and dispensed into 96-well plates at 100 µL per well. Plates were incubated overnight at 4°C to facilitate antigen coating. After a single wash with PBS-Tween (phosphate-buffered saline containing 0.5% Tween 20), positive and negative control sera, as well as test sera from sheep, were diluted 1:100 and added to the wells as the primary antibody at 100 µL per well. Following another wash, HRPO-conjugated recombinant Protein A/G (Pierce 32490) was diluted 1:20000 in PBS-Tween and added to the wells. Following the additional washing steps, 100 µL of chromogenic substrate, comprising 2 µg of ortho-phenylenediamine in 0.1 M citrate buffer at pH 5.5 with 0.03 % $H_2O_2$, was added to each well. Plates were incubated at room temperature for 15 minutes. The reaction was terminated by adding 100 µL of 4 $NH_2SO_4$ to each well. Absorbance was measured at 490 nm using an automated ELISA reader (VERSAmax, USA). The ELISA cut-off values were defined as the mean optical density (OD) of negative sera plus three standard deviations (SD). To determine these values, each reference-positive serum was tested ten times via ELISA, each negative serum five times, and each brucellosis-positive serum ten times. The mean and standard deviation of the results were subsequently calculated.

**Statistical analysis**

Percentages were calculated using descriptive statistics in Microsoft Excel for Microsoft 365 (Microsoft Corporation, version 2024).

## RESULTS AND DISCUSSION

According to the history and clinical findings, there were no previous diagnoses of brucellosis in the herds, and no cases of abortion, lameness, or orchitis were observed. The history indicated no signs of disease in the sheep herds. Additionally, clinical examinations of the sampled sheep exhibited no signs of illness or infection.

The use of R-iELISA on serum samples from sheep in Istanbul, Kocaeli, and Çanakkale yielded 15 positive results (10%), of which eight were confirmed positive by S-iELISA. Consequently, eight sera demonstrated positivity in both R-iELISA and S-iELISA assays. All positive findings marginally exceeded the established cut-off thresholds, indicating borderline results. All results from the slide and tube agglutination analyses were negative. Since eight serum samples tested positive on both S-iELISA and R-iELISA, these positive results were associated with smooth cell structures. Conversely, the positive results exclusive to R-iELISA were associated with rough cell structures. All breeds yielded positive results, yet this positivity was limited to sheep from just three provinces in Turkey. The serological findings are summarized in Table 2.

**Table 2.** Serum samples testing for ELISA, categorized by province of origin, sheep breed, collection period, and associated test results in Turkey

| No | Sheep Breed | Province | Date | R-iELISA | S-iELISA | S-RBPT | R-RBPT | S-TAT | R-2ME-TAT |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Merino | İstanbul | March 2022 | P | P | N | N | N | N |
| 2 | Merino | İstanbul | March 2022 | P | P | N | N | N | N |
| 3 | Lacaune | İstanbul | May 2022 | P | P | N | N | N | N |
| 4 | Lacaune | İstanbul | May 2022 | P | P | N | N | N | N |
| 5 | Merino | İstanbul | May 2022 | P | P | N | N | N | N |
| 6 | Kıvırcık Cross | İstanbul | November 2022 | P | N | N | N | N | N |
| 7 | Merino Cross | Kocaeli | July 2022 | P | N | N | N | N | N |
| 8 | Merino Cross | Kocaeli | July 2022 | P | N | N | N | N | N |
| 9 | Merino Cross | Kocaeli | July 2022 | P | N | N | N | N | N |
| 10 | Merino Cross | Kocaeli | January 2023 | P | N | N | N | N | N |
| 11 | Merino Cross | Kocaeli | January 2023 | P | N | N | N | N | N |
| 12 | Kıvırcık | Çanakkale | March 2022 | P | N | N | N | N | N |
| 13 | Kıvırcık | Çanakkale | March 2022 | P | P | N | N | N | N |
| 14 | Kıvırcık | Çanakkale | March 2022 | P | P | N | N | N | N |
| 15 | Kıvırcık | Çanakkale | January 2023 | P | P | N | N | N | N |

P: Positive, N: Negative, iELISA: İndirect enzyme-linked immunosorbent assay, RBPT: Rose Bengal plate test, TAT: Tube agglutination test

*Brucella melitensis* is the primary causative agent of small ruminant brucellosis, which can lead to significant economic losses in sheep and goat farming (WOAH, 2025a). Because it is also zoonotic, it can infect humans and is recognized as the dominant etiologic agent in human brucellosis (Çelik et al., 2023). *Brucella ovis* infects only rams, causing ovine epididymitis, and affects ewes, leading to infertility and culling in sheep flocks (WOAH, 2025b). *Brucella melitensis* and *B. ovis* are etiological agents that cause economic losses worldwide (WOAH, 2025a;b). The present study randomly sampled rams from different provinces and flocks, thereby improving diagnostic evaluation. Notably, Galluzzo et al. (2021) observed seropositivity predominantly in males among both rams and ewes in a *B. ovis*-positive flock, as detected by R-iELISA and the complement fixation test (CFT). The present results were consistent with the findings of Galluzzo et al. (2021), implying that male animals were more likely to yield positive serological findings.

The CFT, double agar gel immunodiffusion (AGID) test, and iELISA are among the most efficient and widely used methods for the serological diagnosis of *B. ovis* (WOAH, 2025b). Standard diagnostic techniques for *B. ovis* have been implemented in numerous countries. Although there is no international standardization, several independent validation studies have demonstrated that iELISA has higher sensitivity compared to the CFT or AGID tests (Nielsen et al., 2004; Praud et al., 2012; WOAH, 2025b). All serological assays used in the present study were among those recommended by the WOAH for detecting antibodies against smooth and rough *Brucella* strains. The iELISA was considered to be a more sensitive technique than other methods and practical for processing large numbers of samples (WOAH, 2025a;b). To detect antibodies produced against smooth *Brucella* strains, standard smooth *Brucella* strains can be used as antigens to coat iELISA plates. Alternatively, only the S-LPS or O-PS layers from the cell walls of these strains can be extracted and used as antigens (WOAH, 2025a). In a large-scale comparative study, Legesse et al. (2023) evaluated 2,317 serum

samples using an iELISA with S-LPS antigen alongside RBPT and CFT, reporting that the iELISA exhibited the highest specificity and sensitivity for diagnosing *B. abortus*. Consistent with these findings and the recommendations of WOAH (2025a), the present study used the standard smooth strain *B. abortus* S99 as the antigen in all tests designed to detect smooth antibodies, whereas the S-LPS layer derived from the same strain was specifically used as the antigen in the iELISA.

Several studies have assessed the effectiveness of ELISA-based methods in diagnosing *B. ovis* infection serologically (Chiarenza et al., 2018; Savić et al., 2018; Elderbrook et al., 2019). Pradu et al. (2012) examined 4,599 ram sera using a commercial iELISA kit and CFT and reported that iELISA exhibited high sensitivity with acceptable specificity, indicating that iELISA could be reliably employed in large-scale epidemiological studies. Similarly, Costa et al. (2016) compared several serological assays for *B. ovis* antibody detection and found ELISA to be the most sensitive. In a broader survey, Elderbrook et al. (2020) tested 2,276 sheep serum samples using two different ELISA techniques, detecting seropositivity rates of 0.88% and 1.10%, respectively, supporting the use of ELISA for population-level screening. More recently, Keskin et al. (2022) developed iELISA assays using four distinct antigenic agents of *B. ovis* and tested them on 84 sheep serum samples. Keskin et al. (2022) found that the results across these assays had no notable differences, indicating that different antigen preparations may produce similar diagnostic results. Consistent with previous studies, the current results demonstrated that the iELISA achieved the highest diagnostic sensitivity among the serological tests used, highlighting its importance as a main screening method for brucellosis in sheep populations.

The WOAH reported that iELISAs targeting S-LPS or O-PS as antigens were highly sensitive in detecting anti-*Brucella* antibodies in cattle, small ruminants, and pigs. It was noted that iELISAs using S-LPS may accidentally detect antibodies against the rough *B. abortus* RB51 vaccine due to shared antigenic structures. Furthermore, the problem of false positivity, especially in pigs, can be mitigated but not entirely eliminated by using iELISAs prepared with extracts from rough *Brucella* strains. Most false positives were reported to result from cross-reactions with the O-PS portion of the S-LPS molecule. Regarding false positivity, the brucellin skin test remains the most specific diagnostic method. Nonetheless, its use was restricted to countries that did not perform vaccination (WOAH, 2025a). The most commonly used method for preparing antigens from the *B. ovis* REO 1982 strain for the serological diagnosis of *B. ovis* infection is the hot saline extract, which obviated the need for carbon dioxide (WOAH, 2025b). The high-water solubility and abundance of cell-surface epitopes explained the excellent performance of *B. ovis* antigens in serological assays.

In areas with *B. melitensis* presence or where sheep were vaccinated with *B. melitensis* Rev.1, the accuracy of *B. ovis* diagnosis was assessed using the results of serological tests for smooth *Brucella* strains (WOAH, 2025b). In the present study as well, sheep sera were tested and evaluated together using different assays with smooth and rough antigens. The positive results observed in S- and R-iELISAs, which were marginally near the cutoff thresholds during the present study, indicated that these sheep may have been exposed, whether recently or historically, via environmental sources or colostrum. However, the probability that the detected antibodies are of maternal origin is minimal, considering the brief half-life of maternal antibodies (Watson, 1992). Several studies have reported that false-positive reactions in serological *Brucella* tests may result from cross-reactions or non-specific agglutination with other Gram-negative bacteria, including certain species of *Yersinia*, *Bordetella*, *Pseudomonas*, *Francisella*, and *Salmonella* (CFSPH, 2025). Additionally, there may be cross-reactions with other bacteria that possess similar R-LPS and S-LPS structures, such as *Dichelobacter nodosus*, *Arcanobacterium pyogenes*, *Corynebacterium ovis* (WOAH, 2025b), and *Yersinia enterocolitica* O:9 (WOAH, 2025a). Hurvell (1972) found serological cross-reactions between antibodies against *Yersinia enterocolitica* O:9 and four *Brucella* species, including *B. abortus*, *B. melitensis*, *B. suis*, and *B. neotomae*, using immunodiffusion and immunoelectrophoresis assays on agar media. All these strains demonstrated similar tendencies to cross-react. However, rough *Brucella* strains (*B. canis* and *B. ovis*) did not exhibit detectable cross-reactions with *Yersinia enterocolitica* O:9. Víctor et al. (2021), comparing crude *B. canis* RM6/66 antigen (CA-iELISA) with the immunodominant GroEL protein (GroEL-iELISA) for canine brucellosis, reported that while both assays demonstrated cross-reactions with *Leptospira interrogans*, the GroEL-iELISA additionally cross-reacted with *Salmonella* spp. In a more extensive evaluation, Yao et al. (2022) assessed iELISAs targeting *Brucella* outer membrane proteins (Omp31, BP26, Omp25) and a multi-epitope fusion protein, using 34 *Brucella*-positive and 62 *Brucella*-negative canine sera. They found that BP26 and Omp31 exhibited high sensitivity but cross-reacted with *Vibrio parahaemolyticus* and *Listeria monocytogenes*, whereas Omp25 demonstrated poor sensitivity and discriminatory ability. The multi-epitope fusion protein, despite exhibiting mild cross-reactions with *Escherichia coli* H7, *Salmonella*, and *Vibrio parahaemolyticus*, exhibited the best overall performance and was identified as the best antigen for worldwide serodiagnosis of canine brucellosis. These findings underscored the importance of selecting appropriate antigens to optimize serological diagnosis. Nielsen et al. (2004) compared serological assays for detecting antibodies against different bacteria and reported that cross-reactions among *E. coli* O157:H7, *Yersinia enterocolitica* O:9, and *B. abortus* could occur depending on the test used. Consequently, extensive cross-reactivity was observed in iELISA,

whereas reactivity was much lower in the fluorescent polarization assay (FPA) and competitive ELISA (cELISA). In the present study, it was considered that all eight positive results obtained by both iELISAs were likely due to exposure to smooth-structured bacterial agents, either *Brucella* or other microorganisms with similar structures. This result was due to S-LPS containing O-PS and shared antigenic structures with R-LPS. In contrast, the eight samples that were positive only on R-iELISA were generally considered indicative of *B. ovis* infection, with a lower likelihood of cross-reactivity with other rough-structured bacteria that share similar epitopes.

Consistent with the present study, which used heterologous antigens from *B. canis* and *B. melitensis* B115, numerous studies have supported the use of heterologous rough *Brucella* strains as test antigens for the serological diagnosis of *Brucella* with rough cell walls. Perletta et al. (2023) demonstrated that serological tests utilizing *B. ovis* antigens are effective for diagnosing *B. canis* in dogs. Additionally, Escobar et al. (2010) found that rough strains of *B. canis*, *B. ovis*, and *B. abortus* RB51 were suitable for diagnosing *B. canis*. Due to shared antigenic structures between *B. ovis* and *B. canis*, ELISAs developed with *B. ovis* antigens can detect *B. canis* infection (López et al., 2006). They noted that *B. canis* antigens could serve as a complementary tool to official tests for diagnosing *B. ovis* in sheep flocks.

## CONCLUSION

The detection of iELISA-positive results near the cut-off threshold, along with age of sheep (≤1 year), indicated a low likelihood of active *Brucella* infection with either smooth or rough strains. However, the detection of low-titer anti-*Brucella* antibodies did not entirely exclude prior exposure to *Brucella* agents or previous infections in these flocks, and the epidemiological importance of this result should be carefully evaluated. The combined use of serological analyses encompassing both smooth and rough cell wall structures was an effective approach, significantly enhancing diagnostic accuracy. Using multiple assays together significantly enhances the accuracy of interpreting results. For the serological diagnosis of brucellosis in sheep, it is strongly recommended to utilize antigens derived from both the smooth and rough cell wall structures. Active surveillance in sheep flocks is essential for identifying and preventing the spread of brucellosis, and future studies should focus on diagnosing brucellosis caused by *B. melitensis* and *B. ovis*. Furthermore, additional studies should be conducted across different regions of Turkey to yield more reliable results.

## DECLARATIONS

### Authors' contributions

Ahmet Murat Saytekin, Hüsamettin Avcı, Ayfer Güllü Yücetepe, Enikö Kiraly-Avcı, Kemal Metiner, and Sevil Erdenliğ Gürbilek have contributed to the investigation, methodology, formal analysis, writing, and reviewing the manuscript. Ramazan Gül and Ayda Nur Aksoy have contributed to the investigation, writing, and editing of the manuscript. All authors have read and approved the final edition of the manuscript.

### Availability of data and materials

All data supporting the findings of this study may be requested upon reasonable justification from corresponding author.

### Competing interests

The authors declared no competing interests.

### Ethical considerations

All authors confirmed that this study is an original article and has not been published before, nor is it under consideration elsewhere. No AI tools were used for preparing and writing the present study. The authors accept the responsibility for the originality of the study in the entire process of publication.

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