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Serological Assays of Brucellosis in Unvaccinated Sheep and Goat in Indonesia

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ABSTRACT

Brucellosis is recognized as one of the most widespread zoonotic diseases and is often overlooked, impacting livestock populations worldwide. Annual routine screenings utilize serological assays, such as the Rose Bengal test (RBT) and the complement fixation test (CFT), to identify the presence of brucellosis. The present study aimed to evaluate the diagnostic and discriminative performance of the CFT, the locally produced RBT antigen from Indonesia, the commercial RBT antigen (Pourquier), and the indirect enzyme-linked immunosorbent assay (ELISA) using sera from Indonesian goats and sheep. A total of 184 serum samples were collected, including 92 from goats and 92 from sheep. The samples were obtained through jugular venipuncture from animals on small-scale goat and sheep farms in Java Island, Indonesia. The results were analyzed to determine true prevalence (TP), accuracy, and cross-tabulation with parallel interpretation to assess the sensitivity and specificity of each test analysis. The TP of brucellosis in small ruminant serum samples using local RBT, Pourquier RBT, CFT, and indirect ELISA was 6.99%, 9.74%, 0%, and 14.78% in goats, and 6.92%, 20.34%, 0%, and 7.81% in sheep, respectively. Moderate Kappa interrater reliability was observed between the local and Pourquier RBTs for both goats (0.537) and sheep (0.440). The combined RBT sensitivity in goats and sheep was 27% and 20%, respectively, while the combined RBT specificity was 92% in goats and 80.5% in sheep. Using more than one diagnostic method is essential for screening and investigating brucellosis in goat and sheep populations. A combination of RBT and indirect ELISA tests is recommended to identify *Brucella* reactors among small ruminants in Indonesia.

Keywords: Brucellosis, Complement fixation test, Indirect enzyme-linked immunosorbent assay, Rose Bengal test, Small ruminant

INTRODUCTION

Brucellosis remains a major global health issue, affecting livestock quality, farmers' economic prosperity, and public health (Zhang et al., 2018). Bacterial disease caused by the genus *Brucella* (*B.*) infects a wide range of species and can potentially be transmitted to humans (Franc et al., 2018). The *Brucella* spp. belong to the facultative intracellular α-2 division of proteobacteria (Poetsch and Marchesini, 2020). Small ruminants are vulnerable to infection by *B. ovis* and *B. melitensis*. Infection with *B. ovis* in sheep leads to decreased fertility, while *B. melitensis* is the most dangerous to humans (Moreno, 2014). Human cases of brucellosis mainly occur from consuming raw milk and dairy products from infected animals, as well as through direct and indirect contact with these animals (Adetunji et al., 2020). *Brucella* spp. can contaminate the carcasses of cattle, sheep, and goats (Casalinuovo et al., 2016). Meat derived from animals infected with *Brucella* poses a risk of disease transmission, particularly if the contaminated product is not cooked thoroughly (Potter, 2013).

Indonesia is a tropical country with a suitable climate for small ruminant farming. According to statistics from Indonesia, in 2022, Indonesia's goat population was 18,560,835, and the sheep population was 14,063,214 (BPS, 2022). The small ruminant population is mainly concentrated in Java Island, accounting for 52.83% of the country's goat population and 91.04% of the sheep population (BPS, 2022). In Indonesia, brucellosis remains endemic in regions such as East Nusa Tenggara, Sulawesi, Java, Sumatra, and Kalimantan (Padaga et al., 2018). Benkirane (2006) suggested that in regions, such as the Mediterranean, as well as Central and Western Asia, where *B. abortus* infection has a high prevalence, *B. melitensis* infections have also been reported in small ruminants. The exact prevalence of goat and sheep brucellosis in Indonesia remains uncertain, likely due to the ongoing focus on brucellosis eradication and control efforts mainly targeting cattle brucellosis. Additionally, the primary reason for this undetected incidence is the limited understanding of the diagnostic methods established for brucellosis in small ruminants.

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Small ruminant brucellosis not only affects animals and public health but also causes significant financial losses due to clinical signs such as reproductive issues, abortions, stillbirths, decreased milk production, and the costs associated with treating and culling infected animals (Abedi et al., 2020). Additionally, managing and treating infected herds requires considerable investment in veterinary care and biosecurity measures. Large-scale vaccination campaigns and test-and-slaughter programs have successfully helped countries, including the United States, Canada, the European Union, and Norway, eliminate brucellosis, particularly in small ruminants (Zhang et al., 2018). However, the program is less effective in areas with high brucellosis prevalence that lack financial resources or veterinary services. Godfroid et al. (2013) noted that low-resource and developing countries need to expand their efforts in brucellosis control. One suggested approach for eradicating the disease is a one-health integrated system for monitoring and surveillance, aiming to prevent brucellosis in both humans and animals.

The prevalence of brucellosis in goats and sheep in Indonesia has been insufficiently studied. A study in 2015 in the Gunung Kidul district, Yogyakarta, Indonesia, revealed that the brucellosis rate in goats was as high as 9.6% (25/260) at the animal level, using serial testing with the Rose Bengal test (RBT) followed by the complement fixation test (CFT; Primatika et al., 2016). Mujiatun et al. (2016) reported a seroprevalence of 0.84% (1/119) for brucellosis in goats from slaughterhouses in Jakarta, Indonesia, as determined by CFT. Additionally, a recent study reported the seroprevalence of brucellosis in a private farm in Bogor, Indonesia, using RBT and CFT, with 22.81% (26/114) in goats and 19.61% (20/102) in sheep (Martindah et al., 2023).

The reference test for diagnosing brucellosis involves directly culturing *Brucella* from tissue samples, which can be challenging due to lengthy cultivation times and the potential for blood culture failure (Geresu and Kassa, 2015). These procedures require advanced biosafety measures. Serological testing is the most common method used to monitor antibodies against *Brucella* spp. in goat and sheep populations to determine the prevalence of brucellosis in these animals (Godfroid et al., 2010). Diagnostic methods, including the RBT and CFT, are widely accepted for identifying brucellosis reactors in cattle. However, few comparative studies have employed different serological tests, such as RBT, CFT, and enzyme-linked immunosorbent assay (ELISA), to distinguish brucellosis in local goat and sheep populations (Ridlo et al., 2024). To identify effective detection techniques for improving brucellosis prevention and control efforts in Indonesia, it is essential to compare different methods for accurately diagnosing brucellosis in goat and sheep herds. Thus, the present study aimed to evaluate the ability to diagnose brucellosis and the discriminative capacity of CFT, as well as the effectiveness of two RBT antigens, an Indonesian-manufactured *Brucella* antigen and a commercial *Brucella* antigen from Pourquier-IDEXX, in distinguishing positive results compared to indirect ELISA.

MATERIALS AND METHODS

Ethical approval

For the present study, goat and sheep owners provided their informed consent. This investigation was approved under ethical clearance code 93/EC-FKH/Int./2023 by the Institutional Review Board, Faculty of Veterinary Medicine, Gadjah Mada University, Indonesia.

Study area and animals

A total of 184 serum samples were obtained, comprising 92 samples from goats and 92 from sheep, collected from October 2023 to February 2024 in East Java Province and Yogyakarta, Indonesia. The animals were kept in a mixed farming system, with sheep and goats housed near cattle. Serum samples were collected from 48 small ruminant farms, including 23 goat farms and 25 sheep farms. Herds and flocks were selected randomly, with 3 to 5 samples collected from each farm. Data were gathered regarding the different breeds of goats, including Peranakan Etawah, Saanen, Sapera, Senduro, Jawa breed, and mixed breeds. In the case of sheep, the predominant breed was identified as the thin-tailed breed. Both male and female animals aged from 6 to 12 months and over one year were included in the study. The average body weight of local goats in Indonesia was reported to be approximately 20-30 kg for females and 25-40 kg for males. For local sheep, the average weight of males and females was found to be approximately 20-30 kg (Susilorini and Kuswati, 2019).

Sample collection

Approximately 4 mL of blood was collected from the jugular vein using a single-use vacutainer tube. The samples were stored at 4°C overnight to facilitate clotting. After centrifuging for ten minutes at 3000 rpm, the serum was separated into three aliquots and transferred to microcentrifuge tubes. Before serology analysis, the serum samples were stored at -20°C (Shabbir et al., 2013). The serum samples underwent serological testing using CFT, RBT, and indirect ELISA. The CFT analysis was conducted at the Disease Investigation Centre in Wates, Yogyakarta, Indonesia. Rose Bengal tests were conducted at the Veterinary Public Health Laboratory, and the indirect ELISA procedure was carried

out at the Veterinary Physiology Laboratory, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia.

Complement fixation test

The CFT serology assay was performed following the method described by the World Organization for Animal Health (WOAH, 2022). The process of inactivating undiluted serum involved immersing it in a water bath at 58°C for 30 minutes. A 25 µL aliquot of CFT buffer was added to all wells of the U-shaped microplate. Serum samples, positive control, and negative control were added to row A, 25 µL each. Serum dilution was performed by transferring 25 µL of inactivated serum from row A to row B, then mixing with buffer in row B wells, and subsequently transferring 25 µL from each row to the next (rows C to H). *Brucella* antigen suspension (BBVET Wates, Indonesia) 25 µL was added to each well in rows B to H. Row A wells received 25 µL of CFT buffer. Next, 25 µL of complement suspension was added to all wells (A to H). Then, the microplate was covered and incubated for 30 minutes at 37°C. Each well received up to 25 µL of sensitized sheep red blood cells 3% (RBCs). Sensitization was achieved by adding hemolysin to coat the sheep RBCs (WOAH, 2022). After covering, the microplate was incubated at 37°C for 30 minutes and then stored in the refrigerator overnight. A positive result was indicated by complete inhibition of hemolysis.

The Rose Bengal test

The local RBT was manufactured in Indonesia, specifically for the *Brucella* antigen (Pusvetma, Indonesia). The antigen was prepared using a suspension of *Brucella* strain 1119 at a concentration of 8% in buffer with a pH of 3.65. Carefully, 30 µL of serum was mixed with 90 µL of *Brucella* antigen (1:3). The plates were manually rotated for four minutes. Any level of clumping or agglutination identified positive results, whereas a negative result indicated no clumping or agglutination (WOAH, 2022). The commercial *Brucella* antigen, Pourquier RBT (Pourquier® Rose Bengal antigen, IDEXX, France), comprised a suspension of *B. abortus* (Weybridge 99 strain). For the test, approximately 25 µL of serum was mixed with 25 µL of antigen on a white plate. The mixture was gently tilted for four minutes at room temperature and then observed. Agglutination signified a positive result.

Indirect enzyme-linked immunosorbent assay

The indirect ELISA was selected as the reference standard for this study due to its high reported sensitivity and specificity for small ruminant brucellosis. Therefore, the commercial goat *Brucella* antibody immunoglobulin-G (IgG) ELISA kit (Bioenzy, Cat. No BZ-08080000-QLEB, China) and a Sheep *Brucella* antibody IgG ELISA kit (Bioenzy, Cat. No BZ-08190000-QLEB, China) were used. According to the manufacturer's guidelines, the test was performed on microtiter plates pre-coated with *Brucella* antigen. Positive and negative controls, each with a volume of 50 µL, were included. Serum samples were diluted in a sample diluent at a 1:50 ratio. Then, 50 µL of each sample was added to the wells and incubated for 30 minutes at 37°C, followed by aspiration and five washes with PBS. Horseradish peroxidase was added to each well, and the plates were incubated for another 30 minutes at 37°C. After five rinses, the tetramethylbenzidine substrate solution was added, and the plates were sealed for 10 minutes at 37°C in the dark. The reaction was stopped by adding 50 µL of acidic stop solution, resulting in a color change from blue to yellow. The optical density (OD) was measured at 450 nm using a spectrophotometer (AMR-100, Allsheng, China). The cutoff values were 0.293 for sheep and 0.448 for goats, calculated using the formula (Cutoff value = average negative control value + 0.15), as recommended by the kit manufacturer (Bioenzy, China). A positive result was indicated when the OD value was equal to or greater than the cutoff.

Data analysis

The sero-prevalences were calculated by dividing the number of positive results from RBT, CFT, and indirect ELISA tests by the total number of serum samples. The apparent prevalence (AP) and true prevalence (TP) for each serological test result were determined using the online calculator Epitools (Sergeant, 2018). Comparison analysis among the serological tests was performed using the receiver operating characteristic (ROC) curve, the area under the curve (AUC) was calculated along with its standard error (SE) and 95% confidence interval (CI; MedCalc Software Ltd, Ostend, Belgium, 2024), and the Kappa value (JASP, 2024). The AUC measures how well a test can distinguish between positive and negative results, where 1 signifies perfect separation and 0.5 indicates no ability to differentiate (Çorbacıoğlu and Aksel, 2023). The interpretation of the Kappa values was as follows. 0 - 0.20 indicated poor agreement, 0.21 - 0.40 indicated fair, 0.41-0.60 indicated moderate, 0.61 - 0.80 indicated proper, and 0.81 - 1.00 indicated perfect agreement. A 2 × 2 contingency table was employed to determine sensitivity (Se) and specificity (Sp). The Se was calculated as p (T+/D+), and Sp as p (T-/D-), according to Trevethan (2017). Both Se and Sp were derived through parallel interpretation. The animals were considered positive for brucellosis if they tested positive on one or both

tests.

RESULTS

Serum samples were collected from dairy goats (Peranakan Etawah, Saanen, Sapera, and Senduro) and meat breeds (Jawa and mixed breeds). The distribution of goat breeds was as follows. Peranakan Etawah (13/92), Saanen (5/92), Sapera (22/92), Senduro (34/92), Jawa (10/92), and mixed breed (8/92). Female goats were more common (68/92) than males (24/92), with fewer young goats (Less than 1 year, 20/92) compared to adults (More than 1 year, 72/92). All sheep serum samples were collected from meat-breed sheep, including thin-tailed sheep (87/92) and mixed-breed sheep (5/92). The number of female sheep was higher than that of males (56/92 and 36/92, respectively), with more adult sheep (71/92) than young sheep (21/92).

The serological analyses revealed that the RBT with Pourquier antigen and IgG indirect ELISA produced the highest positive results, followed by the local RBT. Table 1 displays the estimated TP for each serological test. The highest detection of *Brucella* in goats (14.78%) was achieved using indirect ELISA, while in sheep (20.34%), it was achieved using Pourquier RBT.

The Kappa analysis was conducted for two RBTs and an indirect ELISA. Table 2 showed a moderate level of agreement between the two RBT antigens used (Local and Pourquier) for goats and sheep, with values of 0.537 and 0.440, respectively. A Kappa value between 0.41 and 0.61 indicated moderate reliability and inconsistency between Local RBT and Pourquier RBT results. Negative Kappa value of -0.098 for Local RBT in sheep indicated poor diagnostic accuracy. The area under the ROC curve between CFT and indirect ELISA results indicated no agreement between goats and sheep (0.500). The CFT demonstrated limited discrimination capacity, potentially attributed to low Se in small ruminants or testing during a chronic infection phase (Sadhu et al., 2015). Furthermore, the combined results of the RBT exhibited poor agreement compared to the indirect ELISA, as the standard reference for brucellosis diagnosis in sheep and goats, in goats and sheep (Table 3, Figure 1). The AUC values for combined RBT in goats and sheep were 0.594 and 0.502, respectively, indicating poor diagnostic accuracy. The Se and Sp of the two RBT antigens were determined using cross-tabulation compared to indirect ELISA (Table 4). The Se of the combined RBT increased in goats and sheep, by 27% and 20%, respectively. In contrast, the Sp decreased for goats and sheep by 92% and 80%, respectively.

Table 1. True prevalence estimation of two RBT antigens, CFT, and indirect ELISA for the sheep and goat sera

Animal	Serology test	(+)/samples	Apparent prevalence		True prevalence	
			Estimate	95% CI	Estimate	95% CI
Goat	Local RBT	6/92	6.52%	0.0148 - 0.1157	6.99%	0.0256 - 0.1583
	Pourquier RBT	8/92	8.7%	0.0294 - 0.1445	9.74%	0.044 - 0.1928
	CFT	0/92	0	0	0	0 - 1.002
	Indirect ELISA	15/92	16.3%	0.0876 - 0.2385	14.78%	0.0793 - 0.2464
Sheep	Local RBT	7/92	7.61%	0.0219 - 0.1303	6.92%	0.0214 - 0.1613
	Pourquier RBT	17/92	18.48%	0.1055 - 0.2641	20.34%	0.1218 - 0.3162
	CFT	0/92	0	0	0	0 - 0.4175
	Indirect ELISA	10/92	10.87%	0.0451 - 0.1732	7.81%	0.0229 - 0.1689

True prevalence was calculated using the online calculator Epitools, source: Sergeant (2018), (+)/samples: Indicates positive results per total sample tested, CI: Confidence intervals, RBT: Rose Bengal test, CFT: Complement fixation test.

Table 2. Kappa value between local RBT, Pourquier RBT, and indirect ELISA results of brucellosis in sheep and goat sera

Animal	Test category	Kappa value	95% CI	Agreement
Goat	Local RBT - Pourquier	0.537	0.211 - 0.863	Moderate
	Local RBT- indirect ELISA	0.107	-0.124 - 0.339	Poor
	Pourquier - indirect ELISA	0.166	-0.084 - 0.416	Poor
Sheep	Local RBT - Pourquier	0.440	0.188 - 0.691	Moderate
	Local RBT- indirect ELISA	-0.098	-0.148 - -0.048	Poor
	Pourquier - indirect ELISA	0.013	-0.187 - 0.213	Poor

RBT: Rose Bengal test, CI: Confidence intervals. The CFT results were excluded from the analysis due to zero results.

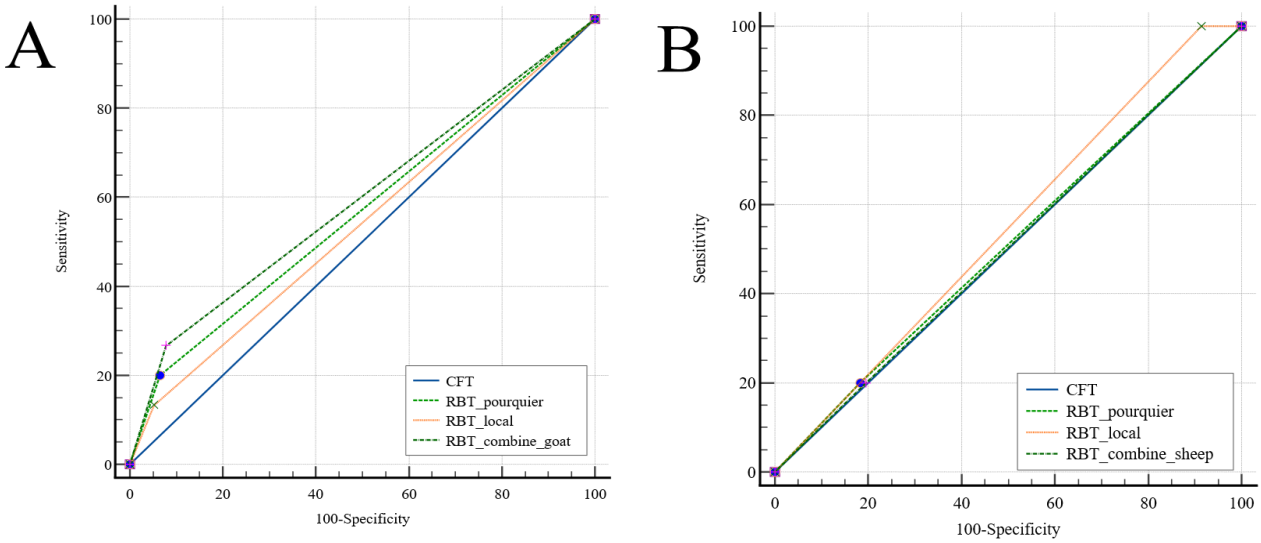

Figure 1. The receiver operating characteristic curve reflects the discriminative ability of CFT, local RBT, and Porquier RBT tests against indirect ELISA for brucellosis in goats (**A**) and sheep (**B**). Both curves show that CFT and RBT have no predictive capability for diagnosing brucellosis in small ruminants.

Table 3. Area under the curve of the receiver operating characteristic curve for CFT, Local RBT, and Pourquier RBT compared to indirect ELISA of brucellosis in sheep and goat serum

Animal	Variable	AUC	SE	95% CI
Goat	CFT	0.500	0.000	0.394 - 0.606
	Pourquier RBT	0.568	0.0553	0.460 - 0.670
	Local RBT	0.541	0.0472	0.434 - 0.645
	RBT goat combined	0.594	0.0611	0.487 - 0.696
Sheep	CFT	0.500	0.000	0.394 - 0.606
	Pourquier RBT	0.509	0.0700	0.402 - 0.614
	Local RBT	0.543	0.0155	0.436 - 0.647
	RBT sheep combined	0.502	0.0702	0.396 - 0.608

AUC: Area under the curve, SE: Standard error, CI: Confidence intervals, RBT: Rose Bengal test, CFT: Complement fixation test, RBT combined: Number of positive results by local RBT, Porquier RBT, or both tests.

Table 4. Sensitivity and specificity of serial interpretation and parallel interpretation of local RBT and Pourquier RBT results compared to the Indirect ELISA of brucellosis in sheep and goat sera

Animal	Serology test	Sensitivity	95% CI	Specificity	95% CI
Goat	Local RBT	13%	0.02 - 0.04	95%	0.87 - 0.99
	Pourquier RBT	20%	0.04 - 0.48	94%	0.85 - 0.98
	RBT combined	27%	0.08 - 0.55	92%	0.84 - 0.97
Sheep	Local RBT	0.0%	0.00 - 0.31	91%	0.81 - 0.96
	Pourquier RBT	20%	0.03 - 0.56	82%	0.72 - 0.89
	RBT combined	20%	0.03 - 0.56	80%	0.70 - 0.88

CI: Confidence intervals, RBT: Rose Bengal test, RBT combined: Positive results by local RBT, Porquier RBT, or both tests.

DISCUSSION

Brucella bacteria can persist for months in materials such as internal organs, abortion tissues, animal excreta, placenta, and products, potentially contaminating the environment (Marvi et al., 2018). Provinces in Indonesia, including Jakarta, West Java, East Java, Yogyakarta, Central Java, Banten, North Sumatra, Aceh, Lampung, Riau, East Nusa Tenggara, West Papua, Central Sulawesi, South Sulawesi, and Maluku, have reported cases of bovine brucellosis (Ridlo et al., 2024). However, there is limited information about brucellosis in sheep and goats, as well as the effectiveness of serology tests. Diagnostic tools for small ruminant brucellosis in Indonesia are limited and often rely on a single test (Martindah et al., 2023). To accurately distinguish between infected and non-infected animals and prevent unnecessary culling due to test errors, highly sensitive and specific tests are necessary. Moreover, technical issues and challenging

biological, epidemiological, and economic factors influence the performance of available tests (Ducrotoy et al., 2018). The current findings provided valuable insights for improving diagnostics in the brucellosis control program among small ruminant populations.

The Peranakan Etawah breed is the most common dairy goat in Indonesia, originating from the crossbreeding of indigenous Kacang breeds with the Etawah breed from India (Sudrajat et al., 2021). The Sapera breed resulted from crossing a female Peranakan Etawah with a Saanen buck (Hermawati and Nuraeni, 2024). The Senduro breed originates from the Senduro district in Lumajang, East Java, and is cultivated for both its milk and meat (Ciptadi et al., 2019). Etawah crossbreed and Sapera goats represented significant advancements in Indonesian goat breeding, focusing on improving milk production and adaptability. Most sheep in Indonesia are meat-type, particularly Javanese thin-tailed sheep, which are essential to the local livestock system (Sodiq and Tawfik, 2004). The present study reported the first case of brucellosis seropositivity in the Senduro goat breed, with the majority of the serum samples analyzed originating from this breed and the thin-tailed sheep. The present investigations, using indirect ELISA, indicated a higher seroprevalence in goats (16.30%) than in sheep (10.87%). False positive results were caused by the RBT using two antigens (Local RBT and Pourquier) in goat and sheep sera, which the indirect ELISA did not detect. Furthermore, the CFT results were negative in all serum samples, which most likely resulted from incomplete or insufficient host immunity caused by early-stage infection or latent infection at the time of sampling (Rossetti et al., 2022). A modified protocol (3:1 ratio of serum to antigen) was used for the local RBT antigen to enhance test sensitivity. Akhter et al. (2014), Tulu et al. (2020), and Šerić-Haračić et al. (2022) indicated that sheep and goats infected with *Brucella* may possess lower antibody levels to the main *Brucella* epitopes compared to cattle. According to Sadhu et al. (2015), the proportions of seropositive small ruminants measured by RBT are frequently higher than those measured by ELISA. Some conditions of the serum samples, particularly contamination and hemolysis, are anti-complementary; therefore, complement cannot be detected in the CFT, and is considered negative despite the presence of antibodies, which supported the findings of the present study as well as Anochie et al. (2023). These anti-complementary reactions produce invalid CFT test results. Additionally, the prozone effect can cause false-negative CFT results in serum with very high antibody titers by forming small, soluble immune complexes that do not precipitate (Thrusfield et al., 2018). Occasionally, blocking antibodies inhibit antigen-antibody interactions (Praud et al., 2012). This frequently happens during CFT for *B. abortus* in cattle, as elevated levels of excess IgG1 can disrupt IgG2, which is essential for complement fixation (Frischauf et al., 2024).

In surveillance studies, serology tests are beneficial for disease screening and detection. However, using a single serology test often results in both false-positive and false-negative outcomes. The TP of the disease is typically not reflected by the seropositive rate, also known as AP (Habibzadeh et al., 2022). The AP, Se, and Sp can all be used to estimate the TP. The results of TP indicated an increase in both RBT antigens used for investigating brucellosis in goats (6.99% and 9.74%), while the AP of local RBT in sheep was lower than the TP (6.92%), and the TP of Pourquier RBT increased (20.34%). Goats exhibited a notably higher prevalence of detected *Brucella* strains than sheep, which aligned with the findings of Hamada et al. (2017), suggesting that immunological responses might differ significantly between species. The TP estimate for CFT in the current study was identical for both sheep and goats, resulting in a zero value. The reference Se for CFT in both goats and sheep was considered low, while the Sp for CFT was 100%, indicating a high Sp, which was consistent with the findings of Rahman et al. (2013) and Legesse et al. (2023). In the present study, indirect ELISA indicated the highest TP rates among serological tests, with 14.78% for brucellosis in goats. The current findings indicated the presence of *Brucella* infection in small ruminants from Lumajang Regency in East Java and Sleman Regency in Yogyakarta, Indonesia. The indirect ELISA offers advantages such as reproducibility, ease of use with minimal equipment, and the availability of commercial kits. Based on the present results, indirect ELISA is a proper alternative to CFT for laboratories with limited resources, which was aligned with the finding of Padilla et al. (2010).

The indirect ELISA result served as the reference test for evaluating the Se and Sp of the RBT and CFT tests. The analysis of CFT, RBT, and indirect ELISA results indicated a lack of agreement between goats and sheep. However, the combined RBT produced the highest AUC score for distinguishing brucellosis in goats. In the present study, the strongest agreement among serology tests was found between two RBT antigens used to detect brucellosis in goats and sheep, with values of 0.537 and 0.440, reflecting a moderate level of agreement. A low Kappa value indicated that the correlation between the two tests was not strong, suggesting that only one test was practical or that both tests were effective but exhibited opposing correlations (Nofuentes and Luna, 2007). The current findings align with those of Praud et al. (2012) and Šerić-Haračić et al. (2022), demonstrating consistency in the accuracy of the testing methods employed, as both tests emphasized the importance of Se and Sp in accurately determining the disease status of animals. This consistency underscores the value of indirect ELISA as a complementary screening tool to RBT, particularly in small ruminant populations, where it has been shown to maintain high sensitivity in detecting brucellosis.

In Asia and Europe, the most commonly used serological tests for official brucellosis control programs are the RBT and the CFT (Godfroid et al., 2010). In the present study, *Brucella* strain 1119 at a concentration of 8% was used. In contrast, in the study by Minas et al. (2008), the whole bacteria, *B. abortus* 19 or 1119-3 cells, served as the antigen in both RBT and CFT tests, while a smooth lipopolysaccharide (LPS) *Brucella* antigen was used in the indirect ELISA test. The indirect ELISA test was selected as the reference test in the present study to determine the Se and Sp of RBT. Similarly, both RBT antigens exhibited higher Se and Sp in detecting brucellosis in goats compared to sheep. The current findings are consistent with Hamada et al. (2017), who indicated that the parallel interpretation method enhances accuracy and effectively manages diagnostic uncertainties. Similarly, Smith et al. (2019) confirmed that the parallel interpretation method significantly boosts confidence in diagnostic results. The current results indicated that combining RBT antigens with cross-tabulated indirect ELISA yielded higher Se for both goat and sheep brucellosis. Parallel testing increased Se but decreased Sp compared to individual RBT, reducing the likelihood of missing a diagnosis of a disease. Several studies by Godfroid et al. (2010) and Chenais et al. (2012) indicated that the RBT may not reliably differentiate between agglutinating reactions caused by *Brucella* species and those elicited by cross-reactive antigens from *Yersinia enterocolitica* O:9. The LPS molecules with similar antigenic epitopes exhibit cross-reactivity among different Gram-negative bacteria, including *Escherichia coli* O116 and O157, *Salmonella enterica* serovar, and *Vibrio cholerae* (Pappas, 2010).

The indirect ELISA may be highly accurate because it can detect minimal amounts of antibodies present during the early stages of infection, whereas RBT was unable to detect them, which was consistent with the study conducted by Erdenebaatar et al. (2004), who demonstrated that indirect ELISA helps to prevent false-positive results caused by non-specific reactor sera that show RBT-positive. Non-clumping antibodies become more dominant than clumping antibodies in the later stages of infections, which may cause them to be undetectable by RBT and CFT (Sha et al., 2025). These non-clumping antibodies can be detected through the addition of an IgG conjugate in an indirect ELISA test. The combination of indirect ELISA and RBT allows investigators to identify every *Brucella* reactor that is seropositive. Using this combined serology test aligns with a study by Padilla et al. (2010), who found that it was not practical to identify every *Brucella* reactor with a single agglutination test. Due to the reliability and reproducibility of the ELISA test, a combination of RBT and ELISA was advised.

Vaccination against small ruminant brucellosis and identifying infected animals through culling or slaughter are effective strategies for reducing its occurrence, spread, and adverse effects (Godfroid et al., 2014). Factors associated with brucellosis risk in small ruminants, according to surveillance data from multiple countries, include breeders' limited knowledge of clinical signs and the absence of a Rev-1 vaccination program for goats and sheep (Coelho et al., 2019). The present results emphasized the importance of utilizing multiple diagnostic methods to accurately identify true positives in livestock populations. These findings are consistent with a study by Elrashedy et al. (2022), who advocated for a multifaceted approach in diagnostic testing to enhance accuracy in livestock health assessments. Such consistency across studies highlighted the critical need for comprehensive diagnostic strategies in veterinary practices.

CONCLUSION

In the current study, indirect ELISA for the local goat and sheep populations in Indonesia was found to be more reliable and sensitive than RBT and CFT. The current results indicated that brucellosis was highly prevalent in the serum samples of goat (16.3%) and sheep (10.87%). It is recommended that a combination of RBT and indirect ELISA be used for the brucellosis surveillance program in small ruminants in Indonesia, as the reliability and reproducibility of the ELISA test enhance the effectiveness of the RBT. The current findings recommend further studies to explore the genetic diversity of *Brucella* spp. in small ruminants in Indonesia and to understand their molecular epidemiology at the national level, thereby guiding the development of economically viable prevention and control strategies.

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Authors' contributions

Ani Setianingrum designed the study, conducted the laboratory analysis, wrote the manuscript, and analyzed the data. Widagdo Sri Nugroho assisted with the conceptualization of the study and interpretation of results. Tri Anggraeni

Kusumastuti revised the manuscript, and Herawati contributed to the literature review. All authors reviewed and approved the last edition of the manuscript.

Competing interests

All authors declared that there is no conflict of interest in the publication of the present study.

Ethical considerations

All authors have confirmed adherence to ethical standards, including topics related to plagiarism, misconduct, fabrication or falsification of data, consent for publication, duplicate publication and/or submission, and redundancy.

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Availability of data and materials

The authors declare that all the necessary data will be provided upon request.

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