RESEARCH ARTICLE

Comparative Evaluation of RBPT and I-ELISA in the Diagnosis of Brucellosis in Small Ruminants and the Analysis of Associated Risk Factors

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Abstract

Brucellosis is a neglected bacterial zoonotic disease affecting livestock and humans in developing countries. Hence, the present study was aimed to determine the seroprevalence of Brucellosis in small ruminants of Tirupati district, Andhra Pradesh, India and to assess the risk factors associated with it. A total of 450 sera samples (245 from sheep and 205 from goats) were screened for *Brucella* antibodies by Rose Bengal Plate Test (RBPT) and by I-ELSIA method. Out of 450 sera samples tested, RBPT detected none of samples positive for the *Brucella* antibodies, while 17 samples (3.77%) showed presence of *Brucella* antibodies by I-ELISA. It was shown that sheep (4.08%, 10/245) had higher infection prevalence than goats (3.41%, 7/205). Risk factor analysis revealed age, abortion history, vaginal discharge, multiparity, mixed farming (sheep and goat together), migratory herds, large herd size, and presence of dogs near farm surroundings, were all found to have a positive statistical association with the seroprevalence of Brucellosis in sheep and goats in the study region. Whereas, sharing of buck, mode of procurement of animals, disposal of aborted materials, and assisting during kidding were found non-significant factors and were not associated with the seroprevalence of the disease in goats. In conclusion, I-ELISA was found to be most sensitive and specific serological test in the diagnosis of small ruminant Brucellosis than RBPT, and there were many risk factors positively associated with its occurrence in the region.

Keywords: Awareness, *Brucella*, I-ELISA, RBPT, Risk factors, Sheep and goats.

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Introduction

Brucellosis is a most widespread and economically devastating contagious bacterial zoonotic disease of sexually matured animals, caused by the genus *Brucella*, a Gram-negative, facultative intracellular bacterium, which lacks capsule, flagella, endospore and native plasmids (Moreno *et al.,* 2002). The different species of *Brucella* affect various vertebrate mammals, *viz*., *B. melitensis* affects sheep and goats, *B. abortus* affects cattle, *B. suis* affects pig, *B. canis* affects dog, and *B. ovis* affects sheep (Scholz *et al.,* 2008). Understanding the occurrence of zoonoses in small ruminants is critical for public health since small ruminants are reservoirs for several zoonotic diseases of important animal and human health concerns (Singh *et al.,* 2018). Seroprevalence studies form the backbone of epidemiological investigation and are used to identify herd infected with *Brucella* (Renukaradhya *et al.,* 2002). The serological tests used for detecting antibodies against *Brucella* organism, *viz.,* Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT), Complement Fixation Test (CFT), Milk Ring Test (MRT) and Enzyme Linked Immunosorbent Assay (ELISA) are simple, affordable and potentially quick. However, none of these tests alone is suitable for all epidemiological studies due to differences in sensitivity and specificity (OIE, 2018).

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RBPT is extensively used as a screening test in herds due to its high sensitivity and has been recognized globally. Even though serological tests like STAT and RBPT are often employed in animals for early Brucellosis screening, it is strongly suggested to add supplemental non-agglutination

testing methods such as ELISA, which is a highly specific and sensitive test for the detection of Brucellosis (Shome *et al.,* 2014). In chronic and convalescent cases, ELISA is more sensitive than STAT and RBPT. Despite the widespread prevalence of Brucellosis among farm animals in India, the major risk factors that precipitate Brucellosis are poorly understood. Therefore, keeping in view these points, the present study was aimed to determine the current status of seroprevalence of Brucellosis in small ruminants of Tirupati district, Andhra Pradesh and to identify risk factors associated with its seropositivity.

Materials and Methods

Collection and Processing of Blood Samples

Following approval of the Institutional Animal Ethics Committee of the College (No. 281/go/ReBi/S/2000/CPCSEA/TPTY/014/VPHE/2023 dated 8.05.23), a total of 450 (245 sheep and 205 goats) blood samples of small ruminants were collected aseptically from different places in Tirupati district by puncturing jugular veins with sterile needles. Samples were collected randomly, from both healthy animals and animals having a history of abortion, retention of placenta, metritis and swelling of the testicles. Approximately, 3-4 mL of blood was collected from each animal in serum clot activator tubes with utmost precaution to avoid haemolysis. The blood collected in serum clot activator tubes was labelled and kept undisturbed for 2-3 h for the serum separation. If the serum was not separated within the prescribed time, then the tubes were centrifuged at 3000 rpm for 2-4 min. The sera samples were stored at -20°C in an ultra-low temperature freezer until tested.

Surveillance using Questionnaire

A semi-structured questionnaire was constructed encompassing individual animal characteristics (age, sex, parity and reproductive disorders), herd characteristics (herd size, herd composition, type of herd, sharing of ram or having own ram and mode of procurement of animals), housing characteristics & biosecurity measures (type of roof/floor, presence of dogs in premises, disposal of aborted material), lambing or kidding practices, disinfection practices followed, animal handler's behaviour (awareness of Brucellosis, consumption of raw milk and farmers assisting during lambing/kidding) and detailed history to investigate the risk factors associated with the occurrence of Brucellosis in sheep and goats of Tirupati district. The responses were collected from animal owners randomly by interviewing while collecting the samples.

Sero-Diagnostic Techniques

All the serum samples were subjected to the Rose Bengal Plate Test (RBPT) and the Indirect ELISA test to identify the prevalence of Brucellosis. The Rose Bengal antigen was procured from the Institute of Veterinary Preventive Medicine (IVPM), Ranipet, Tamil Nadu and the RBPT was carried out as described by Alton *et al.* (1975). Based on the degree of agglutination reaction, the serum samples were considered positive, and its absence was considered negative.

Indirect Enzyme-Linked Immunosorbent Assay (I-ELISA) was done by using an Indirect ELISA kit for sero-diagnosis of sheep and goat Brucellosis, standardized at NIVEDI, Yelahanka, Bengaluru as per the protocol outlined in the user manual supplied with the kit. The degree of colour developed (optical density measured at 492 nm) in this test is directly proportional to the amount of antibody present in the sample. The diagnostic interpretation was made by comparing the optical density (OD) of the test samples with the OD of the control sera samples.

Statistical Analysis

The categorical data of serological tests, *viz*., RBPT and I-ELISA were expressed as percentages by using MS Excel sheet. Risk factor analysis was performed to identify risk factors associated with Brucellosis infection by subjecting the data to Chi-square test.

Results and Discussion

Comparative Efficacy of Serological Tests for Brucellosis

(A) Rose Rose Bengal Plate Test (RBPT)

Out of 450 sera samples (245 from sheep and 205 from goats) collected from different farms of Tirupati district when screened by RBPT revealed that none of the sera samples showed antibodies against *Brucella* (Fig. 1) (Table 1).These results were in concordance with the findings of Madan *et al.* (2022) from Pondicherry, and Sameer (2021) from Maharashtra, who also reported zero prevalence of *Brucella* antibodies by RBPT, both in sheep and goats. Contrary to the present findings, Kanani *et al.* (2018) and Yuguda *et al.* (2022) reported varying seroprevalence rates by RBPT, *viz*., 4.06% in sheep and 7.79% goats in Gujarat, and 4.97% in Chennai.

Table. 1: Overall seroprevalence of Brucellosis in small ruminants by RBPT & I-ELISA

Species	Samples tested	RBPT		I-ELISA	
		No. positive	% positive	No. positive	% positive
Sheep	245	0	0	10	4.08
Goats	205	0	0	07	3.41
Total	**450**	**0**	**0**	**17**	**3.77**

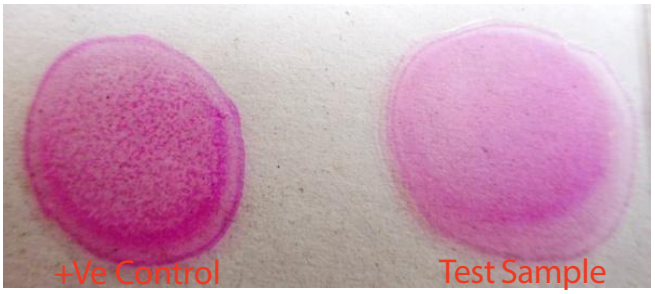

Fig. 1: Rose Bengal plate test (RBPT) for detection of *Brucella* antibodies

The Rose Bengal test is considered as second most sensitive method for detecting reactors in cattle, buffalo, and humans; but it is less effective for detecting Brucellosis in small ruminants (Thakur and Thapliyal, 2004). However, because of cost consideration, feasibility and reliability as a field diagnostic test, RBPT is much cheaper, easier and more convenient to perform than other alternative diagnostic tests (Kanani *et al.,* 2018). The negative results of RBPT in the present study may be due to the use of *B. abortus* antigen which was designed for the diagnosis of bovine Brucellosis; limited sensitivity of RBPT antigen may result in cross-reactions and the test may fail to detect noticeable agglutination, resulting in false negatives. To overcome this, OIE has recommended the usage of 25 µL of the coloured antigen and 75 µL of the blood serum, instead of the standard 1:1 protocol (Blasco *et al.,* 1994). Therefore, the diagnosis of Brucellosis mainly relies on the use of two or more diagnostic tests to confirm the presence of *Brucella* infection.

(B) Indirect Enzyme-Linked Immunosorbent Assay (I-ELISA)

Among 450 sera samples (245 sheep and 205 goats) screened by I-ELISA, 17 samples (3.77%) were found positive for the presence of *Brucella* antibodies (Fig 2). The higher numbers of I-ELISA-positive animals were observed in sheep (4.08%, 10/245) than in goats (3.41%, 7/205), indicating that the two species do not share a similar level of susceptibility to the infection (Table 1). The present findings of I-ELISA were in agreement with the report of Abdalla *et al.* (2019), who recorded a seroprevalence rate of 4.5% (25/558 sera samples) among sheep, while Shome *et al.* (2015) reported 5.5% and 2.3% prevalence in sheep and goats, respectively. Comparable findings were also reported by Natesan *et al.* (2021) and Meena *et al.* (2023). Sadhu *et al.* (2015) from Gujarat reported somewhat higher seropositivity of 11.75% in sheep and 6.02% in goats by I-ELISA; Patel *et al.* (2017) also identified a prevalence of 10.97% and 4.39% in sheep and goats by I-ELISA. In contrast, Abnaroodheleh *et al.* (2021) found much higher seroprevalence in goats (52.2%) than in sheep (16.8%). Similarly, Manasa *et al.* (2019) found a prevalence of 20.45% and 22.7% in sheep and goats by I-ELISA and cELISA in Andhra Pradesh and Telangana regions of India, which is quite high than the results of present study.

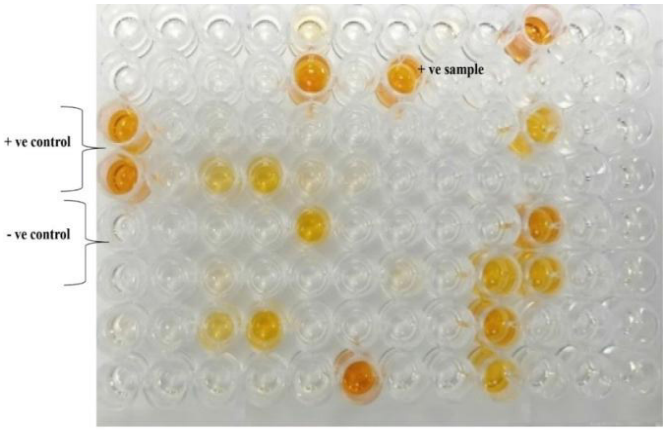

Fig. 2: Microtitre plate showing the result of I-ELISA for detection of Brucella IgG antibodies in sheep and goats

In the present study, I-ELISA was found to be more sensitive and specific serological test in the diagnosis of Brucellosis than RBPT. This finding was in line with Madan *et al.* (2023), who also reported negative results by RBPT and found I-ELISA as a high sensitive test than RBPT. However, Patel *et al.* (2017) reported higher seroprevalence by RBPT (8.70%) than I-ELISA (7.41%) and so also Sadhu *et al.* (2015) found a higher seroprevalence of 11.30% by RBPT and 8.80% by I-ELISA. The difference in test results can be due to a variety of factors, such as the stage of infection, the presence of animals that test false positive or negative and cross-reacting organisms. This may also be due to the antigen used in RBPT being *B. abortus,* whereas the pathogenic agent in sheep and goats is usually *B. ovis* and *B. melitensis,* respectively. Furthermore, various animal species may respond to *Brucella* infection in different ways. It's also possible that different species of immunoglobulins react differently to the tests used to detect Brucellosis. Additional research on these parameters is required, using a large number of sample sizes in both controlled and uncontrolled conditions.

Sex-wise Seroprevalence

The present study found a higher seropositivity rate by I-ELISA in female sheep and goats (4.29%) than in males (2.41%). I-ELISA showed 8 out of 173 female sheep (4.62%) and 2 out of 72 (2.78%) male sheep positive for Brucella antibodies, while in goats, 6 out of 153 females (3.92%) and 1 out of 52 males (1.92%) tested positive. These results were in concordance with the findings of Meena *et al.* (2023) and Vakamalla *et al.* (2023), who also reported higher seropositivity in female sheep and goats than males. Contrary to the present study, Suryawanshi *et al.* (2016), and Natesan *et al.* (2021) reported higher seroprevalence of Brucellosis in male sheep and goats than in females. The higher prevalence of Brucellosis in females could be due to factors, *viz.,* large number of female animals being reared and more samples being collected from females than from males; natural

breeding with Brucellosis-infected male animals presents a high risk of disease transmission in farms. The higher frequency in females could also be attributed to the presence of erythritol content in the placenta that promotes growth and establishment of *Brucella* organisms in the gravid uterus. In the present study area, the lower seroprevalence in males may be due to the eating preference of humans towards male animals, hence the early and frequent removal of male animals from the flocks.

Age-wise Seroprevalence

The present study found 0.95% and 6.43% seropositivity rates in sheep below and above 3 years of age, respectively, and almost similar was the rate for goats also(0.95% and 6.00% in age below and above 3 years) by I-ELISA (Table 2).These findings were similar to the results reported by Suryawanshi *et al.* (2016). Age is the most important factor of Brucellosis to be considered while discussing the seroprevalence of the disease because the risk of disease is more closely related to age than any other factor. The present findings also concord with the studies of Meena *et al.* (2023) and Vakamalla *et al.* (2023), who also reported a higher prevalence in adult animals than younger animals. The higher seroprevalence of Brucellosis found in adults than in younger animals in the present study concurred well with Radostits *et al.* (2007) also stated that sexually mature and pregnant animals were more susceptible to *Brucella* infection than sexually immature animals.

Risk Factors Analysis of Brucellosis

Individual animal risk factors:

The age, sex, parity status, and reproductive history of each animal were considered to study the association of occurrence of Brucellosis. It was shown that sheep had a higher seroprevalence (4.08%) than goats (3.41%). However, there was no statistically significant difference suggesting that the two species do not share the same level of susceptibility to the infection. Similar to this, Jabary and Al-Samarraee (2015) in Iraq found the prevalence of Brucellosis to be 35.2% in sheep and 19.2% in goats and Patel *et al.* (2017) also detected a prevalence of 10.97% in sheep and 4.39% in goats in North-Gujarat. In contrast, the prevalence of Brucellosis in goats was higher than that of sheep in Iran (Abnaroodheleh *et al.,* 2021).

The seroprevalence was higher in adult animals aged above three years than the younger animals below three years and showed significant difference for both sheep and goats ($p<0.05$). These results were similar to the findings of Meena *et al.* (2023), who reported a higher occurrence of brucellosis in a group aged more than 4 years than in the below 4 years age group of sheep and goats. This was also supported by many other studies (McDermott and Arimi, 2002) which recorded that younger animals were more resistant to infection than adult sheep and goats for passive immunisation of younger ones through colostrum of their infected mothers. In this study, vaginal discharge was observed as a significant risk factor ($p<0.01$) in both sheep and goats, with higher odds of being seropositive for Brucellosis. A history of abortion was observed as another risk factor with statistical significance ($p<0.05$) in both sheep and goat flocks, which was in concordance with the results reported by Abnaroodheleh *et al.* (2021) and Alhamada *et al.* (2017).

Herd-level risk factors:

The association of the occurrence of Brucellosis in sheep and goats associated with herd characteristics are represented in Fig. 3. A greater percentage of seropositivity (8.97% and 8.0%) was seen in sheep and goats tested from larger herds than that of smaller herds. The large flock size (>100 animals) was also shown to be a risk factor with higher odds of being seropositive in both sheep ($p<0.01$) and goat flocks ($p<0.05$) than in herd size less than 100 animals. These observations were similar to the findings of Gompo *et al.* (2021). The larger herds, which are likely to be associated with intensive management techniques that allow for closer interaction between animals and their surroundings leading to a higher risk of exposure to infectious excretions, and other factors that facilitate a higher probability of disease spread (Coelho *et al.,* 2008). Sheep and goats reared in mixed farming had higher seropositivity and higher disease risk. This study also revealed that the rearing of mixed herds comprising sheep and goats was a significant risk factor ($p<0.05$) for small ruminant Brucellosis and concurred with the report of Alhamada *et al.* (2017).

Table. 2: Age-wise seroprevalence of Brucellosis in sheep and goats by I-ELISA

Species	Age(years)	Number of Samples tested	I-ELISA	
			No. of positive	% positive
Sheep	<3 years	105	01	0.95
	>3 years	140	09	6.43
Goat	<3 years	105	01	0.95
	>3 years	100	06	6.00

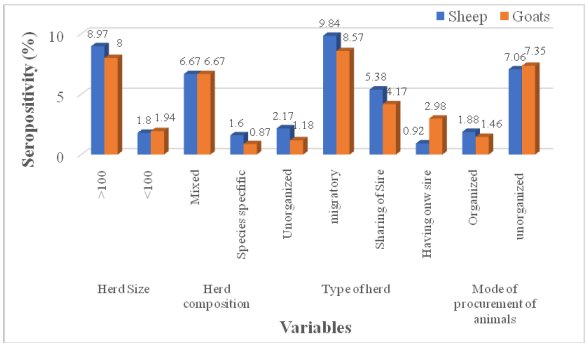

Fig. 3: Association of brucellosis in sheep and goats with herd-level risk factors

Among the herd type characteristics, *viz.,* unorganized herd and migratory herd, the migratory pattern-raised sheep and goats had a higher seropositivity than the unorganized herd type. The pattern of rearing small ruminants as migratory herds was shown to be a significant risk factor in both sheep ($p<0.05$) and goat flocks ($p<0.01$).This was in concordance with results reported by Gompo *et al.* (2021). This may be due to contact with other potentially infected sheep and goats, with other domestic and wild animals during their transit, which enhances disease transmission. Additionally, the herds sharing a ram or buck had a higher seropositivity rate compared to the herds that maintained their own rams or buck. Whereas the mode of procurement of animals either by own flock-raised or purchased-stock has shown minimal significance in the occurrence of Brucellosis.

Housing characteristics

Housing parameters like the type of roof were shown to be non-significant variables. Biosecurity factors such as dog presence surrounding the flocks and method of disposal of aborted materials were taken as risk factors for Brucellosis (Fig. 4). The presence of dogs on the farms also showed a significant association with Brucellosis, with higher odds in goats ($p<0.05$) than in sheep ($p<0.05$). These findings were similar to the observations of Natesan *et al.* (2021). Dogs provide a possible epidemiological concern in Brucellosis-endemic areas because they can serve as mechanical disseminators by consuming aborted foetuses, dragging them along and spreading the bacterium.

Further, the herds following the open method of disposal of aborted materials showed higher seropositivity than the herds following the burial method. It was also observed that the disposal method of aborted materials showed higher odds ($p<0.05$) in sheep than in goats ($p>0.05$). This could be due to the spread of infection by the farm animals or any stray animals such as dogs dragging the aborted foetuses which may contain up to $>10^9$ *Brucella* organisms per gram. Therefore, it is recommended that the aborted foetuses and their contents be disposed of by incineration. Another alternative way is to bury them deep in slaked lime away from farms and waterways (WHO, 2006).

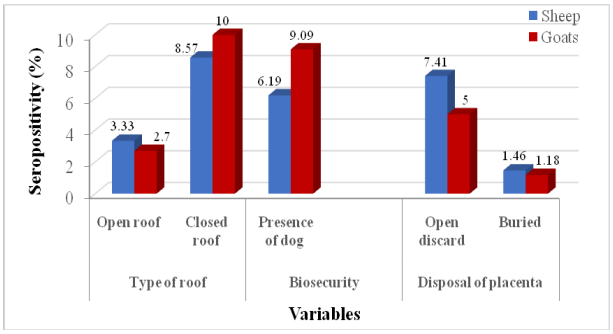

Fig.4: Association of brucellosis in sheep and goats with housing characteristics

Owner-level risk factors:

Owner-level risk factors, *viz.,* awareness of Brucellosis (1.11% each), consumption of raw milk (4.62 and 8.70%) and assistance during lambing (7.21 and 2.31%) were recorded in sheep and goats, respectively. The findings in this study suggest the importance of proper farm management practices like appropriate disposal of aborted materials, vaginal discharges, presence of dogs, migratory flocks, improper assisting of animals during kidding/lambing and mishandling of aborted materials and retained placenta might contribute to the further spread of Brucellosis to humans. Consequently, there is a high risk of pathogen transmission between animals and from animals to humans through direct contact with contaminated materials such as foetal membranes, aborted foetuses and other animal products.

Conclusion

In the present study, Rose Bengal Plate Test (RBPT) was found to be less sensitive in the diagnosis of small ruminant Brucellosis than I-ELISA. The study suggests that a standardized *B. melitensis* and *B. ovis* coloured antigens must be developed for serological diagnosis of Brucellosis in small ruminants such as RBPT. The findings of the current study point towards the fact that risk factors with statistical significance that were associated with high prevalence of brucellosis were age, multiparity, abortion history, vaginal discharges, large herd size, mixed rearing (sheep and goats together), migratory herds, sharing of sire, mode of procurement of animals, presence of dogs in farms, disposal of aborted materials and assisting during lambing were all found to have positive association with statistical significance in sheep and goats.

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