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Seroprevalence and molecular confirmation of brucellosis in sheep from Telangana with phylogenetic analysis of circulating *Brucella* spp.

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Abstract

Background: Brucellosis is an economically important zoonotic disease affecting small ruminants worldwide, causing reproductive losses and posing significant public health concerns. The movement of sheep under government-subsidized livestock distribution schemes may facilitate the spread of infection and increase disease transmission among flocks.

Methods: A cross-sectional study was conducted in sheep farms established under government-subsidized schemes in Telangana, India. Thirteen farms were initially surveyed, and ten farms with a history of reproductive disorders suggestive of brucellosis were selected for investigation. A total of 245 sheep serum samples were screened for *Brucella* antibodies using the Rose Bengal Plate Test (RBPT). Whole blood samples from RBPT-positive animals (n=31) were subjected to genus-specific polymerase chain reaction (PCR) using F4/R2 primers targeting the 16S rRNA gene. Representative PCR-positive amplicons were sequenced and analyzed phylogenetically.

Results: The overall seroprevalence of brucellosis was 12.65% (31/245) by RBPT. Sex-wise prevalence was 16.7% in rams and 12.2% in ewes, whereas age-wise prevalence was highest in sheep aged 1-2 years (14.7%). Lambing-wise analysis showed the highest seropositivity among first-lambing ewes (14.2%). However, no significant association was observed between seroprevalence and sex, age, or lambing status ($p > 0.01$). PCR analysis confirmed *Brucella* DNA in 5 (16.12%) of the 31 RBPT-positive samples by amplification of a 905 bp fragment. Sequence analysis demonstrated high homology with *Brucella* spp. sequences available in GenBank. Phylogenetic analysis revealed close genetic relatedness of the study isolate with globally reported *Brucella* strains while indicating regional divergence among Indian isolates.

Conclusion: The present study confirms the occurrence of brucellosis among sheep maintained under subsidized farming schemes in Telangana. The combined use of RBPT and PCR proved useful for serological screening and molecular confirmation of infection. The detection of *Brucella* DNA and the phylogenetic relationship of the isolate with globally circulating strains highlight the need for continuous surveillance, molecular monitoring, and strengthened biosecurity measures to control brucellosis and reduce its zoonotic risk.

Keywords: Brucellosis, Sheep, RBPT, PCR, Seroprevalence, Phylogenetic analysis, Telangana, *Brucella* spp.

Introduction

Brucellosis is one of the most important zoonotic bacterial diseases affecting livestock and humans worldwide. It is caused by members of the genus *Brucella*, which are Gram-negative, non-motile, non-spore-forming, facultative intracellular coccobacilli belonging to the family *Brucellaceae* (Leclercq *et al.*, 2020) [25]. The disease affects a wide range of domestic animals, including cattle, sheep, goats, pigs, and wildlife species, resulting in significant reproductive and economic losses (Franc *et al.*, 2018) [17].

Brucellosis remains a major public health concern, particularly in developing countries where livestock farming constitutes an important source of livelihood. In humans, the disease is characterized by intermittent or undulant fever, headache, weakness, excessive sweating, weight loss, joint pain, and other systemic manifestations (Pal and Jain, 1986; Addis, 2015; Pal, 2018) [30, 41]. Human infection commonly occurs through the consumption of unpasteurized milk and milk products or through occupational exposure to infected animals and their

secretions (Al-Sherida *et al.*, 2020; Zewdie *et al.*, 2020) [7, 45]. In India, *Brucella melitensis* biovar 1 is responsible for the majority of human brucellosis cases and contributes substantially to economic losses and public health burden (Kumara *et al.*, 2015; Singh *et al.*, 2018) [23, 40].

India possesses one of the largest sheep populations in the world, estimated at 74.26 million, with Telangana accounting for approximately 25.67% of the national sheep population (GOI, 2023). Sheep farming plays a vital role in the socio-economic development of rural communities, particularly among small and marginal farmers. However, productivity in sheep production systems is constrained by several factors, including infectious diseases, among which brucellosis is of considerable importance. The disease is associated with abortions, infertility, retained placenta, reduced fertility, decreased lambing rates, and consequent economic losses.

The transmission of ovine brucellosis occurs primarily through ingestion of contaminated feed and water, inhalation of infected aerosols, and direct contact with aborted fetuses, placental tissues, and uterine discharges from infected animals (Radostits *et al.*, 2007) [34]. Movement of infected animals between flocks and across geographical regions significantly contributes to disease dissemination (Bait *et al.*, 2019) [11]. Pregnant animals are particularly susceptible because the presence of erythritol in the placenta promotes rapid multiplication of *Brucella* organisms, resulting in placentitis, endometritis, and abortion, especially during late gestation (Bait *et al.*, 2019) [11].

Accurate diagnosis is essential for surveillance and control of brucellosis. Isolation and identification of *Brucella* organisms from tissues, milk, or vaginal secretions remain the definitive diagnostic method; however, culture techniques are labour-intensive, time-consuming, and pose a significant biosafety risk to laboratory personnel (Surucuoglu *et al.*, 2009; Pérez-Sancho *et al.*, 2013) [44, 33]. Consequently, serological assays are widely employed for epidemiological investigations and herd-level screening. Commonly used serological tests include the Rose Bengal Plate Test (RBPT), Enzyme-Linked Immunosorbent Assay (ELISA), and Lateral Flow Assay (LFA), owing to their simplicity, rapidity, and cost-effectiveness (Nielsen, 2002) [28].

Molecular diagnostic techniques have further enhanced the detection and characterization of *Brucella* species. Polymerase Chain Reaction (PCR)-based assays targeting genes such as 16S rRNA, outer membrane proteins (OMP), IS711 insertion sequence, and the *BCSP31* gene have demonstrated high sensitivity and specificity for rapid identification of *Brucella* organisms (Baily *et al.*, 1992; Leal-Klevezas *et al.*, 1995; Costa *et al.*, 1996; Londhe *et al.*, 2013; Gupta *et al.*, 2014; Ali *et al.*, 2015) [10, 24, 16, 26, 21, 6]. These molecular tools facilitate reliable diagnosis and provide valuable information for epidemiological investigations.

Control of brucellosis relies on a combination of surveillance, biosecurity measures, and vaccination. In India, the National Control Programme on Brucellosis (NCPB) promotes regular surveillance and vaccination strategies for disease control. The live attenuated *Brucella melitensis* Rev-1 vaccine is widely recommended for the prevention of brucellosis in sheep and goats (OIE, 2004). In Telangana, large-scale sheep distribution programmes and the frequent procurement of animals from neighbouring

states have substantially increased the movement of sheep across different geographical regions. Such animal movements may facilitate the introduction and dissemination of *Brucella* infection into previously unaffected flocks. Despite the large sheep population and the economic importance of sheep farming in the state, comprehensive information regarding the prevalence of brucellosis, associated risk factors, and the molecular confirmation of infection in organized and field sheep flocks remains limited. The lack of updated epidemiological data hampers the development of effective disease surveillance and control strategies. Therefore, the present study was undertaken to determine the seroprevalence of brucellosis in sheep flocks of Telangana using serological tests and to confirm the presence of *Brucella* infection through molecular techniques. The findings of this study will provide baseline epidemiological information for strengthening brucellosis surveillance, prevention, and control programmes in the state.

Materials and methods

Study area, study design and target population

A cross-sectional study was conducted in sheep farms established under various government-subsidized sheep distribution schemes in Telangana State, India. Information regarding the farms was obtained from the Department of Animal Husbandry, Government of Telangana. A preliminary survey was conducted in 13 sheep farms using a structured questionnaire to collect baseline farm-level information and identify flocks with a history suggestive of brucellosis, including abortion, retained placenta, stillbirths, infertility, and testicular swelling.

Based on clinical history and field observations, 10 farms were selected for further investigation. The study aimed to determine the seroprevalence of brucellosis in sheep and to molecularly confirm the presence of *Brucella* spp. in seropositive animals.

Sample size determination

The minimum sample size required to estimate the seroprevalence of brucellosis was calculated using the formula:

$$n = Z^2P(1-P)/d^2 \text{ (Charani et al., 2010 and Islam et al., 2021) [13, 22].}$$

In this formula,

Z represents 1.96, P represents the expected seroprevalence, and d denotes the desired precision set at 5%.

An expected prevalence of 11.55% reported by Shome *et al.* (2021) [39] was used for the calculation, yielding a minimum sample size of 158 animals. To improve the precision and reliability of the prevalence estimates and to compensate for potential sample loss or invalid test results, the sample size was increased to 245 sheep.

Collection and screening of blood samples from sheep

A total of 245 blood samples comprising 221 ewes and 24 rams were collected from the selected farms (Figur 1& Table 1). Blood samples (5 mL) were aseptically collected from the jugular vein using sterile vacutainer tubes containing clot activator. Samples were transported to the laboratory under refrigerated conditions and centrifuged for serum separation. The separated sera were stored at -20 °C until further analysis.



Fig 1: Collection of blood samples from sheep

Table 1: No. of blood samples collected from sheep farms

SL.NO	Farm	Ewes	Rams	Total
1	Farm 1	14	1	15
2	Farm 2	27	3	30
3	Farm 3	25	3	28
4	Farm 4	18	2	20
5	Farm 5	23	3	26
6	Farm 6	22	1	23
7	Farm 7	20	2	22
8	Farm 8	24	3	27
9	Farm 9	16	1	17
10	Farm 10	32	5	37
Total		221	24	245

Serological screening by Rose Bengal Plate Test (RBPT)

All serum samples were screened for antibodies against *Brucella* spp. using the Rose Bengal Plate Test (RBPT) according to the method described by Alton and Jones (1967) ^[8] and Alton *et al.* (1988) ^[9]. The Rose Bengal antigen (*Brucella abortus*) was procured from the Institute of Animal Husbandry and Veterinary Biologicals (IAH&VB), Hebbal, Bengaluru.

Briefly, equal volumes (30 μ L each) of serum and antigen were mixed thoroughly on a clean glass slide and observed for visible agglutination within 4 minutes. Samples exhibiting agglutination were considered positive, while those showing no agglutination were considered negative. Blood samples from RBPT-positive animals were subsequently subjected to molecular analysis (Figure 2).

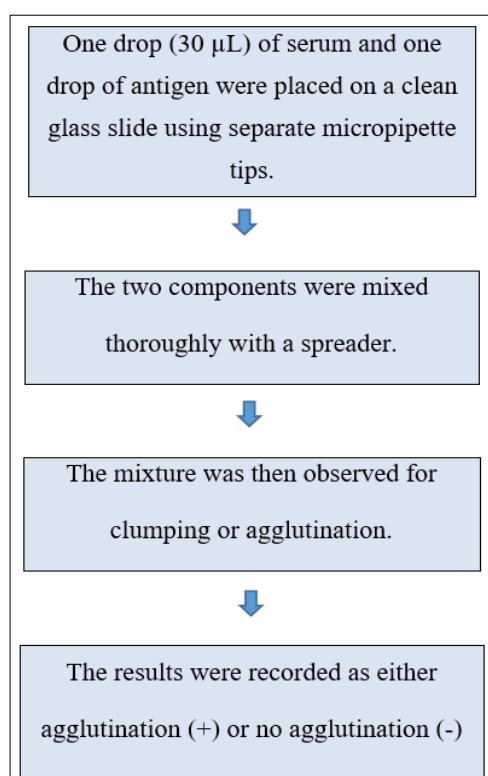


Fig 2: Flow diagram for screening of serum samples for brucellosis

Genomic DNA extraction

Genomic DNA was extracted from whole blood samples following a modified phenol-chloroform extraction protocol described by Leal-Klevezas *et al.* (1995) [24]. Briefly, erythrocytes were lysed using RBC lysis buffer followed by leukocyte lysis and protein digestion using Proteinase K. DNA was purified using phenol: chloroform alcohol (25:24:1), precipitated with isopropanol and sodium acetate, washed with 70% ethanol, and finally resuspended in TE buffer. Extracted DNA samples were stored at 4 °C until further use.

DNA quantification

The concentration and purity of extracted DNA were determined using a NanoDrop spectrophotometer. DNA quality was assessed by measuring absorbance at 260 and 280 nm. Samples with an A260/A280 ratio of approximately 1.8 were considered suitable for PCR amplification (Shirish, 2021) [38].

PCR amplification of *Brucella* DNA

Genus-specific PCR targeting the *Brucella* cell surface protein (*BCSP31*) gene was performed using F4 and R2 primers as described by Çiftci *et al.* (2017) [15]. PCR reactions were carried out in a final volume of 25 µL containing 2.5 µL of 10× PCR buffer, 2 µL MgCl₂ (25 mM), 0.5 µL dNTP mix (10 mM), 1 µL each of forward and reverse primers (10 pmol/µL), 0.6 µL Taq DNA polymerase (5 U/µL), 8 µL template DNA, and nuclease-free water to make up the final volume.

Amplification was performed in a Prima-DUO® thermal cycler under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 6 min.

Agarose gel electrophoresis

PCR amplicons were resolved by electrophoresis on 1% agarose gel prepared in 1× TBE buffer. Electrophoresis was carried out at 80-100 V for approximately 60 min. Following electrophoresis, gels were visualized under a UV transilluminator (Vilber BioPrint, France) and photographed. Samples producing a specific amplicon of 903 bp were considered positive for *Brucella* spp.

Sequencing and phylogenetic analysis

Representative PCR-positive amplicons were purified and commercially sequenced by Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad. Raw sequence data obtained in ABI format were edited and assembled using MEGA version 12.1 software. The resulting consensus sequences were subjected to Basic Local Alignment Search Tool

(BLAST) analysis against sequences available in the NCBI GenBank database to determine sequence homology and confirm species identity. Phylogenetic relationships were inferred using reference sequences retrieved from GenBank.

Statistical analysis

The apparent seroprevalence of brucellosis was calculated as the proportion of RBPT-positive animals among the total animals tested. Descriptive statistics were used to summarize farm-level and animal-level data. Confidence intervals for prevalence estimates were calculated at the 95% confidence level.

Results

In the present study, Information on sheep farms established under government-subsidized sheep distribution schemes was obtained from the Department of Veterinary and Animal Husbandry, Telangana. A total of 13 sheep farms were identified, and baseline information regarding farm management practices, reproductive health status, and disease history was collected through farmer interviews, farm observations, and consultation with field veterinarians. Based on the presence of clinical signs and reproductive disorders suggestive of brucellosis, including abortion, retained placenta, stillbirths, infertility, and testicular swelling, 10 farms were considered suspected for brucellosis and selected for further investigation. From these farms, 245 sheep were randomly sampled for serological and molecular screening. Screening of suspected sheep for brucellosis Based on the sheep exhibiting reproductive disorders suggestive of brucellosis, including abortion, retained placenta, stillbirths, infertility, and testicular swelling, were selected for laboratory investigation. A total of 276 samples comprising 245 serum samples and 31 whole blood samples were collected from ten sheep farms located in different districts of Telangana. Serum samples were screened for *Brucella*-specific antibodies using the Rose Bengal Plate Test (RBPT), and whole blood samples from seropositive animals were subjected to molecular confirmation by PCR. Representative PCR-positive amplicons were further sequenced and analyzed for phylogenetic relationships.

Seroprevalence of brucellosis in sheep by RBPT

Out of 245 serum samples screened by RBPT, 31 samples were positive for *Brucella* antibodies (Figure 3), resulting in an overall seroprevalence of 12.65% (Figure 4).

Farm-wise analysis revealed variation in seropositivity among the investigated flocks. The highest seroprevalence was recorded in Farm 5 (23.1%), followed by Farm 2 (16.7%), Farm 8 (14.8%), Farm 3 (14.3%), and Farm 6 (13.0%). The lowest prevalence was observed in Farm 9 (5.9%) and Farm 1 (6.7%) (Table 2).

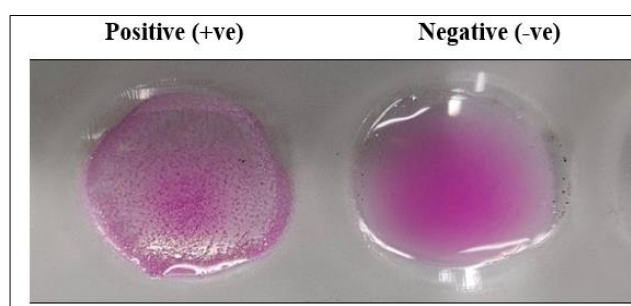
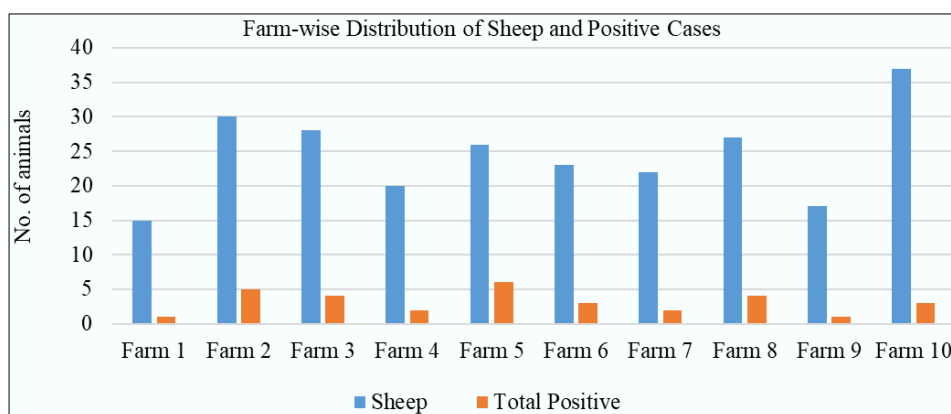


Fig 3: Detection of *Brucella* antibodies by RBPT

Table 2: Overall Seroprevalence of brucellosis in sheep by RBPT

Sl.No	Farm	Sheep	Total positive	Positivity (%)
1	Farm 1	15	1	6.70%
2	Farm 2	30	5	16.70%
3	Farm 3	28	4	14.30%
4	Farm 4	20	2	10.00%
5	Farm 5	26	6	23.10%
6	Farm 6	23	3	13.00%
7	Farm 7	22	2	9.10%
8	Farm 8	27	4	14.80%
9	Farm 9	17	1	5.90%
10	Farm 10	37	3	8.10%
Total		245	31	12.65%

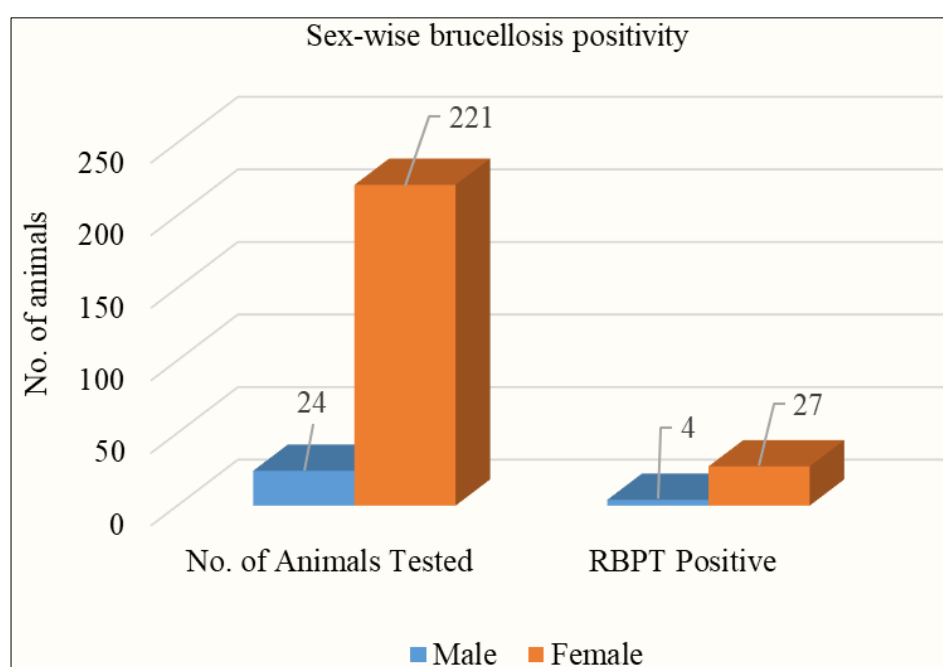
**Fig 4:** Distribution of positive cases among sheep farms**Sex-wise prevalence**

Among the 221 ewes tested, 27 animals were seropositive, corresponding to a prevalence of 12.2%. In contrast, 4 of the 24 rams tested were positive, yielding a prevalence of

16.7% (Table 3; Figure 5). Although seropositivity was numerically higher among rams, the association between sex and seroprevalence was not statistically significant ($p > 0.01$).

Table 3: Sex-wise prevalence of brucellosis in sheep

Sex of the animal	No. of animals tested	RBPT		p-value
		+ve	%	
Male	24	04	16.6	>0.01
Female	221	27	12.2	

**Fig 5:** Sex-wise distribution of RBPT positive sheep

Age-wise prevalence

Age-wise analysis indicated that the highest seroprevalence was observed in sheep aged 1-2 years, where 19 of 129 animals (14.7%) tested positive. In the 2-4 years age group, 9 of 85 sheep (10.5%) were positive, while 3 of 31 sheep older than 4 years (9.6%) were seropositive (Table 4; Figure 6). Despite the higher prevalence observed in younger animals, no statistically significant association was detected between age and seropositivity ($p > 0.01$).

Table 4: Age-wise prevalence of brucellosis in sheep

Age	No. of sheep tested	RBPT		p-value
		+Ve	%	
1-2 years	129	19	14.7	>0.01
2-4 years	85	9	10.5	
> 4 years	31	3	9.6	

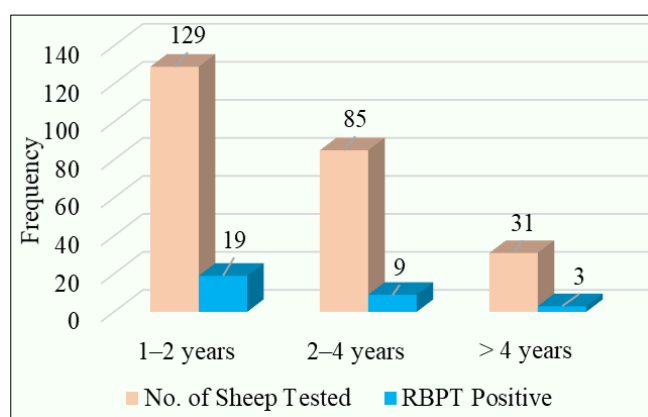


Fig 6: Age-wise Distribution of RBPT Positive Sheep

Lambing-wise prevalence

Among ewes, seropositivity varied according to lambing status. Sheep in their first lambing exhibited the highest prevalence, with 17 of 119 animals (14.2%) testing positive. The prevalence decreased to 10.2% (8/78) among animals in their second lambing and to 8.3% (2/24) among ewes with more than two lambings (Table 5; Figure 7). However, these differences were not statistically significant ($p > 0.01$).

Table 5: Lambing-wise prevalence of brucellosis in sheep

Lambing	No. of ewes tested	RBPT		p-value
		+Ve	%	
First lambing	119	17	14.2	>0.01
Second lambing	78	8	10.2	
More than two lambings	24	2	8.3	

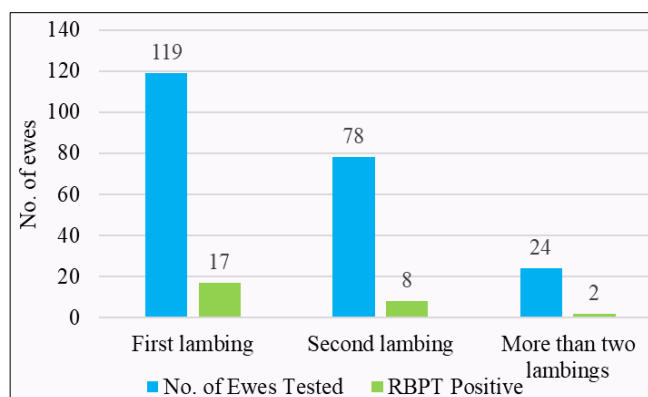


Fig 7: Lambing-wise distribution of RBPT positive ewes

Molecular detection of *Brucella* spp. by PCR

All 31 RBPT-positive samples were subjected to genus-specific PCR using the F4/R2 primer pair targeting a 905 bp fragment of *Brucella* DNA. Before sample screening, PCR conditions were optimized using gradient PCR. Among the annealing temperatures evaluated (52-60 °C), 60 °C produced a distinct and specific amplification product of the expected size without non-specific bands and was therefore selected for subsequent analyses.

Of the 31 RBPT-positive blood samples examined, 5 (16.12%) yielded the expected 905 bp amplicon, confirming the presence of *Brucella* DNA (Figure 8). The remaining 26 samples (83.88%) were negative by PCR. Of the five PCR-positive animals, four were ewes and one was a ram (Table 6).

Farm-wise distribution of PCR-positive cases indicated the presence of molecularly confirmed brucellosis in four farms. Two positive cases were detected in Farm 10, whereas Farms 2, 4, and 8 each contributed one positive case (Table 6).

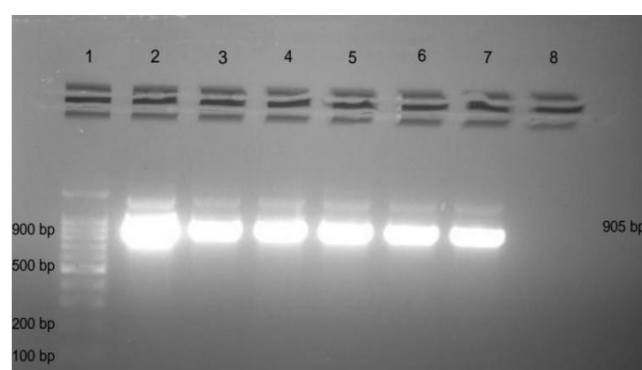


Fig 8: PCR amplification of *Brucella* spp. (905 bp) using F4/R2 Primers; Lane 1: 100-bp DNA ladder; Lane 2: positive control; Lanes 3, 4, 5, 6 and 7: positive samples (905 bp); Lane 8: negative control

Table 6: Confirmation of brucellosis by PCR in seropositive sheep

SI.No	Farm	RBPT positive Ewes	RBPT positive Rams	PCR positive Ewes	PCR positive Rams	Total PCR positive
1	Farm 1	1	0	0	0	0
2	Farm 2	4	1	1	0	1
3	Farm 3	3	1	0	0	0
4	Farm 4	2	0	1	0	1
5	Farm 5	5	1	0	0	0
6	Farm 6	3	0	0	0	0
7	Farm 7	2	0	0	0	0
8	Farm 8	4	0	1	0	1
9	Farm 9	1	0	0	0	0
10	Farm 10	2	1	1	1	2
Total		27	4	4	1	5

Sequencing and phylogenetic analysis

One representative PCR-positive amplicon was subjected to nucleotide sequencing. The obtained sequence was edited and assembled using MEGA version 12.1.2 and subsequently analyzed using the BLAST algorithm available in the NCBI database. Sequence analysis confirmed homology with previously reported *Brucella* sequences, thereby validating the PCR results.

Phylogenetic analysis demonstrated that the study isolate, designated VPHE/PVNRTGVU/2025, clustered within the *Brucella* lineage and exhibited close genetic relatedness to

previously reported *Brucella* isolates from different geographical regions (Figure 9). The phylogenetic tree revealed distinct clustering patterns among *B. melitensis* and *B. abortus* isolates. The Indian isolates formed a separate branch with strong bootstrap support, indicating regional genetic divergence. The study isolates also showed phylogenetic affinity with strains reported from West Asian

countries, including Syria, Turkey, Jordan, and Afghanistan, suggesting possible transboundary dissemination and genetic connectivity among circulating *Brucella* strains. The sequence generated in the present study contributes to the understanding of the molecular epidemiology and genetic diversity of *Brucella* circulating in sheep populations of Telangana.

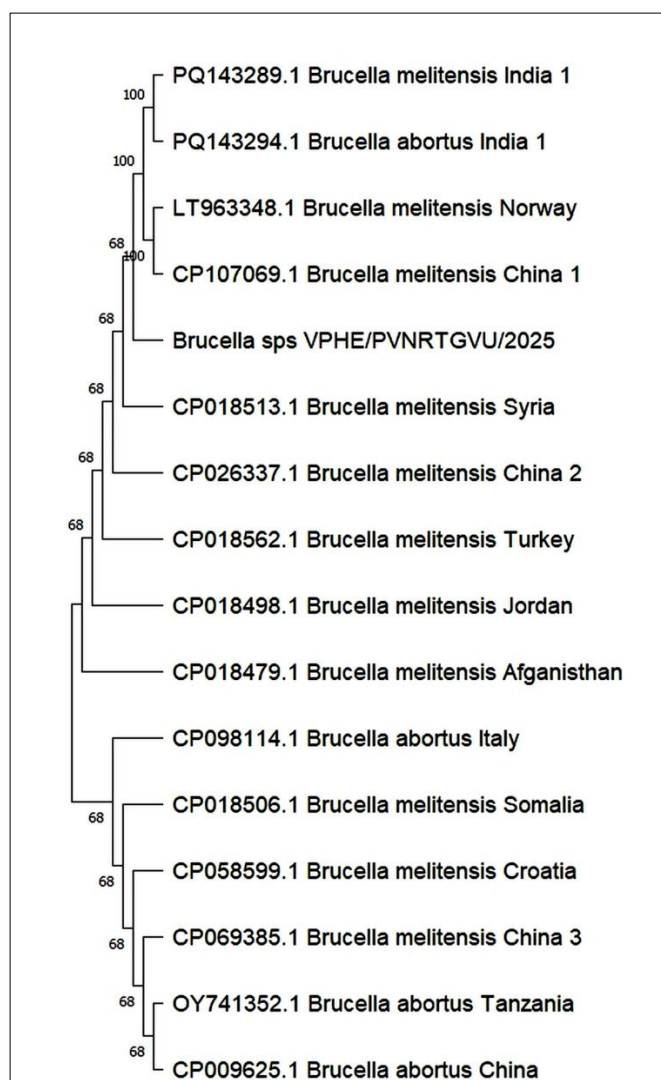


Fig 9: Phylogenetic tree showing genetic relationships among *Brucella* spp. based on partial gene sequences.

Discussion

Seroprevalence of Brucellosis in Sheep

The present study revealed an overall seroprevalence of 12.65% (31/245) in sheep screened by the Rose Bengal Plate Test (RBPT), indicating the continued circulation of *Brucella* infection among sheep populations in Telangana. The observed prevalence suggests that brucellosis remains an important reproductive and zoonotic disease in small ruminants and may pose a potential risk to farmers, animal handlers, and veterinarians.

The seroprevalence recorded in the present study is comparable with reports from Egypt (16.3%) (Selim *et al.*, 2019) [37], Qatar (16.7%) (Al-Hussain *et al.*, 2024) [5], Tunisia (16.1%) (Guesmi *et al.*, 2023) [20], Nepal (15%) (Gompo *et al.*, 2021) [19], and various regions of India where prevalence ranging from 8.4% to 15.5% has been reported in sheep using RBPT and ELISA-based assays (Chauhan *et al.*, 2022; Suresha *et al.*, 2022; SreeLakshmi *et al.*, 2024) [14, 43, 42]. However, the prevalence observed in the present study

was higher than reports from Sudan, Ethiopia, Somalia, and Jammu & Kashmir, where prevalence ranged from 0.18% to 8.92% (Abdalla *et al.*, 2019; Zewdie, 2020; Bile *et al.*, 2024; Mehak *et al.*, 2019; Parthiban *et al.*, 2019) [1, 45, 12, 27, 32]. Conversely, lower prevalence than the present study has been reported from highly endemic areas of Egypt, Iraq, Andhra Pradesh, and Tamil Nadu, where prevalence exceeded 25% and reached up to 60% in some investigations (Abdeltwab *et al.*, 2019; Abdulhameed *et al.*, 2020; Reddy, 2022; Sowmiya *et al.*, 2024) [2, 3, 35, 41].

Variations in prevalence among different studies may be attributed to differences in geographical location, management practices, flock size, animal movement, climatic conditions, sample size, disease endemicity, and diagnostic methods employed. Studies utilizing ELISA-based assays often report higher prevalence because of their greater sensitivity compared with RBPT. Furthermore, investigations involving animals with abortion history or

reproductive disorders generally yield higher prevalence than studies involving apparently healthy animals. Sex-wise analysis revealed a higher seropositivity in rams (16.7%) than in ewes (12.2%), although the difference was not statistically significant ($p > 0.01$). Similar observations have been reported by Selim *et al.* (2019) [37], suggesting that sex may not be a major determinant of infection. The lack of statistical significance in the present study may also be influenced by the relatively small number of rams included compared with ewes.

Age-wise analysis indicated the highest seroprevalence among sheep aged 1-2 years (14.7%), followed by animals aged 2-4 years (10.5%) and >4 years (9.6%). However, the differences among age groups were not statistically significant ($p > 0.01$). Although several studies have reported higher prevalence among older animals due to prolonged exposure and reproductive maturity, the present findings suggest that infection may occur irrespective of age in endemic flocks where continuous exposure to the pathogen exists.

Lambing-wise analysis demonstrated a higher prevalence among first-lambing ewes (14.2%) compared with second-lambing (10.2%) and multiparous animals (8.3%). However, no significant association was observed between lambing status and seropositivity. The relatively higher prevalence in first-lambing animals may be associated with physiological stress during the reproductive period, which could increase susceptibility to infection.

The moderate seroprevalence observed in the present study may be associated with the large-scale movement of sheep under government-subsidized distribution programmes and the introduction of animals from neighbouring states. Such practices facilitate the dissemination of *Brucella* infection between flocks and may contribute to the persistence of the disease in the region.

Molecular Detection of Brucellosis by PCR

Molecular analysis of RBPT-positive samples using genus-specific PCR confirmed the presence of *Brucella* DNA in 5 (16.12%) of 31 seropositive animals. The successful amplification of the expected 905 bp product confirmed active infection and demonstrated the usefulness of PCR as a confirmatory diagnostic tool.

The PCR positivity observed in the present study is comparable with previous reports from sheep and goats, where positivity ranging from 15% to 27% was reported using genus-specific PCR assays targeting the 16S rRNA and *bcsp31* genes [Çiftci *et al.*, 2017; Shirish, 2021; Sankhe *et al.*, 2023] [15, 38, 36]. The relatively moderate detection rate may be attributed to intermittent bacteremia and the low concentration of bacterial DNA in peripheral blood, which can reduce PCR sensitivity.

The lower PCR positivity compared with serological positivity observed in the present study is expected because antibodies may persist long after infection, whereas PCR detects only the presence of bacterial DNA at the time of sampling. Similar discrepancies between serological and molecular results have been reported in previous studies of brucellosis in small ruminants.

Differences in PCR detection rates reported by various authors may be attributed to variations in sample type, target gene, assay sensitivity, and disease stage. Studies utilizing milk, placental tissues, fetal membranes, or aborted materials frequently report higher detection rates because

these tissues contain greater bacterial loads than blood samples. Likewise, real-time PCR assays often demonstrate superior sensitivity compared with conventional PCR methods.

The optimization of PCR conditions in the present study, particularly the annealing temperature of 60 °C, resulted in specific amplification without non-specific products, thereby improving assay reliability and reproducibility. These findings support the use of PCR as an effective adjunct to serological screening for confirmation of brucellosis in sheep.

Sequencing and Phylogenetic Analysis

Sequencing of the representative PCR-positive amplicon confirmed its identity as *Brucella* spp., showing high sequence similarity with reference sequences available in the NCBI database. The high degree of homology observed in the present study is consistent with the conserved nature of the targeted genomic region and agrees with previous molecular investigations conducted in different geographical regions.

Phylogenetic analysis revealed that the study isolates clustered closely with globally reported *Brucella melitensis* and *Brucella abortus* strains, indicating substantial genetic conservation within the genus. At the same time, the formation of distinct clusters among Indian isolates suggests the existence of regional genetic divergence. Similar phylogenetic patterns have been reported in molecular epidemiological studies from Asia, Africa, Europe, and the Middle East.

The close relationship between the study isolates and strains originating from neighbouring and geographically distant countries highlights the potential role of animal movement, trade, and transboundary transmission in the dissemination of brucellosis. Given the extensive movement of sheep associated with breeding and government-supported livestock distribution programmes, such factors may contribute to the circulation and maintenance of *Brucella* strains within the region.

Overall, the sequencing and phylogenetic findings provide molecular confirmation of *Brucella* infection and contribute valuable information on the genetic diversity and epidemiology of circulating strains. These data emphasize the importance of integrating molecular characterization with conventional surveillance programmes to improve understanding of disease transmission dynamics and to support effective brucellosis control strategies.

Conclusion

The present study demonstrated an overall seroprevalence of 12.65% for brucellosis among sheep maintained under government-subsidized farming schemes in Telangana, indicating the continued circulation of *Brucella* infection in the region. Seropositivity was observed across different age groups, sexes, and lambing categories, although no statistically significant association was found between these factors and disease occurrence.

Molecular confirmation by PCR detected *Brucella* DNA in 16.12% of RBPT-positive animals, highlighting the utility of PCR as a reliable confirmatory diagnostic tool for identifying active infection. Sequencing and phylogenetic analysis confirmed the identity of the isolate as *Brucella* spp. and revealed close genetic relatedness with globally reported strains, suggesting possible transboundary

dissemination and emphasizing the importance of molecular epidemiological surveillance.

The findings indicate that sheep movement associated with livestock distribution programmes and the introduction of animals from neighbouring regions may contribute to the maintenance and spread of brucellosis. Therefore, regular serological surveillance, molecular confirmation, screening of replacement stock, farmer awareness programmes, strict biosecurity measures, and strategic vaccination should be strengthened to reduce disease transmission and minimize the zoonotic risk posed by brucellosis to livestock owners, animal handlers, and veterinarians.

The present study provides baseline epidemiological and molecular information on ovine brucellosis in Telangana and may serve as a useful reference for future disease control and eradication programmes.

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