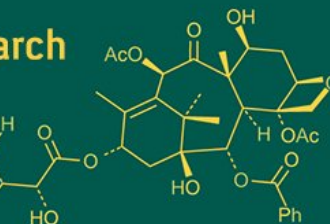
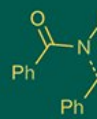


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RBPT and IS711 PCR-Based detection of bovine brucellosis in Tripura, India

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Abstract

Brucellosis is a major bacterial zoonosis causing substantial economic losses to India's livestock sector and posing a significant public health risk, yet its diagnosis remains challenging due to the limitations of bacterial culture, the gold-standard method. This study aimed to detect bovine brucellosis using the Rose Bengal Plate Test (RBPT) as a serological screen alongside PCR targeting the IS711 insertion sequence as a molecular tool. A total of 45 clinical samples (blood, vaginal discharge, aborted foetal organs, and placenta) were collected from cattle with a history of abortion in West Tripura, all previously confirmed seropositive by RBPT. An IS711-based PCR assay was optimized and applied to screen the samples, using DNA from *Brucella abortus* S19 vaccine strain as positive control, yielding a characteristic 497 bp amplicon. Comparative analysis showed 18 samples (40.0%) positive by both RBPT and PCR and 21 (46.7%) negative by both, indicating good overall concordance between the two methods. However, discrepancies were noted in six samples: four RBPT-negative samples tested PCR-positive, while two RBPT-positive samples tested PCR-negative, likely reflecting early-stage infection undetected serologically, residual antibodies from past exposure, or cross-reactivity/sample-related variability, respectively. Overall, PCR demonstrated higher sensitivity than RBPT in detecting *Brucella* infection, particularly in animals with low or undetectable antibody titres. These findings confirm the continued circulation of *Brucella abortus* in bovine populations of Tripura and support the combined use of serological and molecular assays for improved diagnostic accuracy. Such an integrated approach is essential for effective surveillance, early detection, and control of bovine brucellosis in this endemic region.

Keywords: Brucellosis, *Brucella abortus*, RBPT, IS711 PCR, cattle, abortion, Tripura.

Introduction

Brucellosis is one of the most widespread bacterial zoonotic diseases worldwide and remains endemic in India, posing a significant threat to both animal and public health. Brucellosis has been eradicated in many developed countries (Europe, Australia, Canada, Israel, Japan, and New Zealand (Geering *et al.*, 1995) [6] but remains endemic in Africa, the Mediterranean, the Middle East, parts of Asia, and Latin America (Refai, 2002) [19]. Brucellosis is caused by bacteria of the genus *Brucella*, which are small, non-motile, aerobic, facultative intracellular, Gram-negative coccobacilli. *Brucella* ability to survive and multiply within host cells and enables persistent infection and evasion of both innate and adaptive immunity (Fichi, 2003) [5]. Several earlier study showed that brucellosis is associated with substantial economic losses in the livestock sector due to reproductive disorders, including abortion, infertility, retained placenta, reduced milk production, and restrictions on the trade of animals and animal products. In India, brucellosis is estimated to cause a median annual economic loss of approximately US\$3.4 billion (Singh *et al.*, 2015) [20], highlighting its considerable impact on livestock productivity and the national economy.

Brucellosis is recognized as one of the most important occupational infectious diseases in humans, particularly affecting those working in slaughterhouses, as butchers, and as veterinarians. Furthermore, the clinical signs of brucellosis can be highly variable, ranging from non-specific, flu-like symptoms (in the acute form) to undulant fever, arthritis, orchitis, and epididymitis. (Hassan *et al.*, 2012) [8] Transmission of *Brucella* occurs through both direct and indirect routes, including horizontal and vertical transmission in bovines. Major sources of infection include aborted fetuses, placental membranes, uterine discharges, In

domesticated animals, the clinical manifestations of brucellosis mainly associated with reproductive pathology. In females, it responsible for the metritis, mastitis, repeat breeding, third-trimester abortion, retained placenta, and reduced milk yield and in males, it manifests through epididymitis, orchitis, and infertility (Radostits *et al.*, 2000) ^[18] The variety of possible transmission routes, combined with its zoonotic nature, makes brucellosis one of the most dreaded and contagious diseases among all zoonotic and reproductive failure diseases in animals. Infected animals shed the organism through milk, urine, and semen. The gravid uterus of infected pregnant animals harbours large numbers of the organism, making reproductive materials particularly important in disease transmission. The definitive diagnosis of brucellosis relies on the isolation and identification of *Brucella spp* (Alton *et al.*, 1988) ^[1]. Bacterial culture remains the gold standard for diagnosis (Qureshi *et al.*, 2024) ^[17]. Samples testing sero-positive on screening assays such as the Rose Bengal Plate Test (RBPT) should be confirmed using the Complement Fixation Test (CFT), the method recommended by the OIE (World Organisation for Animal Health) to support disease eradication efforts (OIE) ^[15] However, it is labour-intensive, time-consuming, and requires stringent biosafety measures because of the zoonotic nature of the pathogen. Consequently, serological tests have gained widespread acceptance (Nazir *et al.*, 2025) ^[13] as practical, economical, and rapid tools for the diagnosis and surveillance of brucellosis in both animals and humans. In recent years, nucleic acid-based detection methods have emerged as valuable alternatives for the diagnosis of brucellosis. Molecular techniques offer high sensitivity and specificity while eliminating the need to handle viable organisms, thereby reducing laboratory-associated risks. Several genetic targets have been employed for the detection and characterization of *Brucella spp.*, including *bcsp31* (Ingle *et al.*, 2023) ^[9], outer membrane protein (*omp*) genes (Gupta *et al.*, 2007) ^[7], and the insertion sequence *IS711* (Paul *et al.*, 2020) ^[16]. A recent study on the diagnosis and differentiation of vaccine strains of *Brucella* confirmed that a single-tube duplex PCR assay is useful for the early and rapid detection and differentiation of *B. abortus* vaccine strain S19 from other *Brucella spp.* in both milk and clinical specimens (Das *et al.*, 2019) ^[3]. Considering the importance of rapid and accurate diagnosis, the present study was undertaken to detect bovine brucellosis using the Rose Bengal Plate Test

(RBPT) as a serological screening method and polymerase chain reaction (PCR) as a molecular diagnostic tool for the detection of *Brucella spp.* in clinical samples.

Materials and Methods

Place of study

The present study was carried out in the Departments of Veterinary Microbiology and Veterinary Pathology, College of Veterinary Sciences and Animal Husbandry, R.K. Nagar, West Tripura, during the period 2024–2025.

Collection of clinical samples

A total of 45 clinical samples, including blood, vaginal discharge, aborted foetal organs, and placenta, were randomly collected from cattle with a history of abortion, from cattle farms in and around Agartala as well as from various organized farms in the West district of Tripura. Based on prior information regarding abortion history, the animals were kept under continuous monitoring, and prior to sample collection, all animals were serologically tested by the Rose Bengal Test (RBT) and confirmed to be seropositive for brucellosis.

Rose Bengal plate test (RBPT)

The coloured antigen required for the RBPT test was purchased from the Indian Veterinary Research Institute, Division of Biological Products, Izzatnagar. The serum samples were processed for the detection of brucella antibodies by RBPT as per WOA, 2009 ^[22].

PCR assays

Bacterial DNA extraction

Genomic DNA was extracted from *Brucella* broth cultures using the boiling method. Briefly, culture aliquots were transferred to microcentrifuge tubes, boiled for 5 min, and immediately snap-chilled on ice (Didugu *et al.*, 2015) ^[4]. The lysate was then centrifuged at 10,000 × g for 5 min, and the resulting supernatant was used as the template DNA for downstream PCR applications.

Primer design

Oligonucleotide primers targeting the *IS711* insertion sequence of *Brucella spp.* were custom-synthesized by Eurofins Genomics India Pvt. Ltd. The primer sequences, lengths, and expected amplicon size are presented in Table 1.

Table 1: Primers used for the *IS711*-PCR assay

Primers name	Sequence (5'–3')	Length (bp)	Amplicon Size (bp)	Reference
IS711-FP	CGTGTCTGCATTYAACGTAACC	22bp	497 bp	Das <i>et al.</i> , 2018
IS711-RP	TCCTCATTGWCAGCACCATATC	22bp		

Optimization of *IS711* PCR assay

DNA extracted from *Brucella* isolates were used as positive controls in this study. PCR amplification was performed in 0.2 ml thin walled PCR tubes using approximately 2 µl of bacterial genomic DNA, 12.5 µl of 2X Green Dream Taq PCR Master Mix (Thermo-scientific, USA), 1 µl each (eryC-FP and eryCRP) primer (10 pmol/µl), 0.5 µl each (*IS711*-FP and *IS711*- RP) primer 10 pmol/µl and the volume was made up to 25 µl with 7.5 µl Nuclease Free water (NFW). The PCR amplification was performed in a thermocycler ((Biorad, USA) with the cycling conditions consisting of initial denaturation at 94°C for 5 min followed

by 35 cycles at 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec followed by a final extension cycle at 72°C for 7 min. A non-template control was run in all the PCR experiments to rule out the possibility of contamination in the samples. The PCR amplified products were resolved on 1.5 % agarose gel in Tris acetate EDTA (TAE) buffer (1X). The agarose gel was stained with ethidium bromide and documented under UV light in a gel documentation system (Molecular Imager® Gel Doc™ XR+System, Bio-Rad, USA).

Screening of clinical samples

Genomic DNA extracted from the *Brucella abortus* S19

vaccine strain was used as the positive control, while nuclease-free water (NFW) was used as the negative control. Forty-five clinical samples comprising of blood, vaginal discharge, aborted foetal organs and placenta, were randomly collected from cattle with a history of abortion, from west district of Tripura were screened by the optimized IS711PCR assay.

Results

A total of 45 bovine clinical samples collected from different locations in Tripura were screened for brucellosis using the Rose Bengal Plate Test (RBPT) (Fig.- 1) and Polymerase Chain Reaction (PCR). Comparative analysis of the two diagnostic methods revealed that 18 (40.0%) samples were positive by both RBPT and PCR, while 21 (46.7%) samples were negative by both tests. Discrepant results were observed in five samples, of which three (6.7%) were negative by RBPT but positive by PCR, whereas two (4.4%) were positive by RBPT but negative by PCR.

Overall, PCR detected a higher number of positive samples than RBPT, indicating its superior ability to identify *Brucella* infection in clinical specimens. The occurrence of PCR-positive but RBPT-negative samples suggests that molecular detection can identify infected animals that may not yet have developed detectable antibody titres or may possess antibody levels below the detection threshold of serological assays.

Molecular confirmation by is711 pcr assay

All samples were subjected to PCR targeting the IS711 insertion sequence, a highly conserved and widely used molecular marker for the detection of *Brucella* spp. The reference strain *Brucella abortus* S19 was included in each assay as a positive control. Amplification of the target sequence yielded the expected 497 bp amplicon in the positive control (*B. abortus* S19) as well as in the PCR-positive clinical samples. The presence of the characteristic 497 bp product confirmed the detection of *Brucella* DNA in the clinical specimens and validated the specificity of the assay (Fig.-2). The results demonstrated the utility of the IS711-based PCR assay as a rapid and reliable molecular tool for the detection of *Brucella* infection in bovine clinical samples from Tripura.

Table 2: Comparative detection of bovine brucellosis in clinical samples by RBPT and IS711-PCR

RBPT Result	PCR Positive	PCR Negative	Total
Positive	18	2	20
Negative	4	21	25
Total	21	23	45

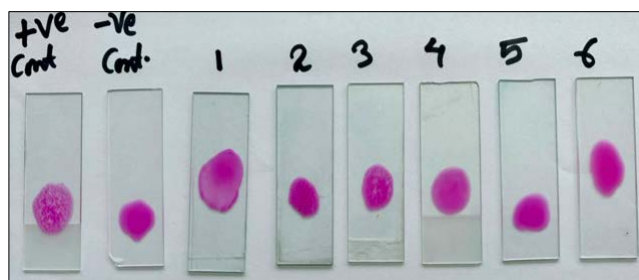


Fig 1: RBPT Tests showing agglutinations of clinical samples both positive RBPT (1, 2 &3) and Negative RBPT (4, 5 & 6)



Fig 2: Amplification of the target sequence yielded the expected 497 bp amplicon in the positive control (*B. abortus* S19) as well as in the PCR-positive clinical samples

Discussion

Brucellosis remains one of the most important zoonotic diseases affecting livestock production and public health worldwide. Rapid and accurate diagnosis is essential for effective disease surveillance and control, particularly in endemic regions such as the North Eastern states of India. In the present study, bovine clinical samples were screened using the Rose Bengal Plate Test (RBPT) and PCR targeting the IS711 gene for the detection of *Brucella abortus*. Due to non-availability of mandatory biosafety laboratory level 3, the desired bacterial isolation and culture was not carried out in this study and hence, for the disease diagnostic purposes agglutination test like RBPT, are routinely performed. Among the 45 clinical samples examined, 18 were positive by both RBPT and PCR, while 21 were negative by both methods, indicating a high degree of concordance between the serological and molecular diagnostic techniques. This agreement suggests that RBPT remains a useful screening tool for the detection of bovine brucellosis under field conditions. However, discrepancies were observed in six samples: four samples negative by RBPT were positive by PCR, whereas two samples positive by RBPT were negative by PCR.

The findings of the present study are consistent with previous reports demonstrating the utility of RBPT as a screening test for brucellosis. Khan *et al.* (2021) [11] screened 220 samples and found 49 positive, corresponding to a prevalence of 22.7%. Similarly, Chand and Chhabra (2013) [12] screened 2,967 samples, of which 663 were found positive. The detection of PCR-positive but RBPT-negative samples highlights the superior sensitivity of molecular methods in identifying *Brucella* infection. PCR detects the presence of bacterial DNA directly in clinical specimens and therefore can identify infected animals even before the development of detectable antibody titres. Such findings may indicate recent or early-stage infections in which the humoral immune response is insufficient to produce a positive serological reaction. Similar observations have been reported by several investigators who demonstrated that PCR is capable of detecting *Brucella* DNA in animals that test negative by conventional serological methods (Kaushik *et al.*, 2006, Neha *et al.*, 2016 and Verma *et al.*, 2017) [10, 14, 21]. The ability of PCR to detect infection at an early stage is particularly important for preventing disease transmission and implementing timely control measures.

In the present study, it has been observed that, two samples were positive by RBPT but negative by PCR. This discrepancy may be attributed to the persistence of antibodies following previous exposure or recovery from infection, resulting in positive serological reactions despite the absence of detectable bacterial DNA. This outcome is in complete in agreement with the earlier study done by

Muñoz, *et al* (2005) [12] and study showed that, false-positive RBPT reactions may occur because of cross-reactivity with other Gram-negative bacteria possessing similar smooth lipopolysaccharide antigens, such as *Yersinia enterocolitica* O:9. Variations in bacterial load, sample quality, DNA extraction efficiency, and the intermittent presence of the organism in clinical specimens may also contribute to negative PCR results in serologically positive animals.

The *IS711*-PCR assay employed in the present study successfully amplified the expected 498 bp product in the positive control (*Brucella abortus* S19) and in PCR-positive clinical samples, confirming the presence of *Brucella* DNA. The *IS711* insertion sequence is widely recognized as a highly specific molecular target because it is present in multiple copies within the *Brucella* genome, thereby enhancing the sensitivity of detection. The successful amplification of the target fragment validates the reliability and specificity of the assay for molecular diagnosis of bovine brucellosis.

Conclusion

This study demonstrates that PCR offers superior sensitivity over RBPT for detecting *Brucella abortus* infection in bovine clinical samples. Nonetheless, RBPT retains considerable value as a rapid, cost-effective, and field-friendly screening tool for large-scale epidemiological surveillance. Integrating serological and molecular diagnostic approaches can substantially enhance detection accuracy and enable earlier identification of infected animals. An approach that is especially critical for the surveillance and control of bovine brucellosis in Tripura and other North-Eastern states of India, where livestock rearing forms a vital component of the rural economy. The continued detection of *B. abortus* in bovine clinical samples from Tripura confirms the ongoing circulation of the pathogen in the region, underscoring the need for intensified surveillance, routine screening programmes, and robust biosecurity measures to curb transmission both among animals and from animals to humans.

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Declaration

Ethics approval and consent to participate

Clinical samples were collected using non-invasive methods, and no animal experiments were conducted. The procedure adopted for sample collection from live animals was approved by the Institutional Animal Ethics Committee (IAEC). All methods were performed in accordance with relevant guidelines and regulations, and the study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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