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R Vivekanandhan

Assistant Professor, Department of Veterinary Public Health and Epidemiology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Udumalpet, Tamil Nadu, India

S Krishna Kumar

Professor and Head, Department of Veterinary Public Health and Epidemiology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Udumalpet, Tamil Nadu, India

M Dharani

Assistant Professor, Department of Veterinary Public Health and Epidemiology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Udumalpet, Tamil Nadu, India

M Boopathy Raja

Assistant Professor, Department of Veterinary and Animal Husbandry Extension Education, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Tirunelveli, Tamil Nadu, India

TA Arul Sabareeswaran

Assistant Professor, Department of Veterinary and Animal Husbandry Extension Education, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Udumalpet, Tamil Nadu, India

Corresponding Author:

R Vivekanandhan

Assistant Professor, Department of Veterinary Public Health and Epidemiology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Udumalpet, Tamil Nadu, India

Comparative sero-diagnostic evaluation of brucellosis among unorganised buffalo farms of Coimbatore, Tamil Nadu

R Vivekanandhan, S Krishna Kumar, M Dharani, M Boopathy Raja and TA Arul Sabareeswaran

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Abstract

Brucellosis is an important zoonotic disease causing reproductive losses in livestock and posing a significant public health concern. This study compared the diagnostic performance of Rose Bengal Plate Test (RBPT), Modified Rose Bengal Plate Test (mRBPT), and competitive ELISA (c-ELISA) for detecting brucellosis in buffaloes from unorganised farms in Coimbatore, Tamil Nadu. A total of 93 serum samples collected from four farms with a history of abortions were screened using all three assays. Overall seropositivity was highest with c-ELISA (54.84%), followed by mRBPT (50.54%) and RBPT (26.88%). Pairwise analysis showed moderate agreement among the tests, while mRBPT and c-ELISA detected significantly more positive animals than RBPT. The mRBPT demonstrated 100% relative sensitivity compared with RBPT, whereas c-ELISA showed high sensitivity and comparable diagnostic performance. The findings suggest that mRBPT is a rapid and reliable field screening test, while c-ELISA is a suitable confirmatory assay for effective brucellosis surveillance in buffaloes.

Keywords: Brucellosis, Buffalo, RBPT, Modified RBPT, c-ELISA, Serodiagnosis

1. Introduction

Brucellosis is an endemic, neglected bacterial zoonotic disease. Small, Gram-negative, facultative intracellular, aerobic coccobacilli of the genus *Brucella* cause brucellosis. In Tamil Nadu, brucellosis is popularly called “Kandruveechunoiy” in Tamil. In areas where agriculture and livestock farming are widely practised, brucellosis is very common. Brucellosis is a major public health concern because close contact between humans and animals in these places can promote its spread (Qureshi *et al.*, 2023) [13]. According to reports, the prevalence of brucellosis in India ranges from 6.5 to 16.4% (Singh *et al.*, 2015; Jaismon *et al.*, 2023) [17,6]. According to Singh *et al.* (2015; 2018) [17,18], brucellosis was responsible for an estimated annual economic loss of US\$3.4 billion in livestock and US\$10.46 million in human health. Brucellosis infected animals have clinical manifestations that include intermittent fever, abortion, retained foetal membrane and reduced milk yield (Ghssein *et al.*, 2025) [4]. Approximately 1.6 to 2.1 million people have been affected every year worldwide (Laine *et al.*, 2023) [7], with potential risk factors including pathogen shedding from non-pregnant animals, lifelong shedding in bulls, and chronic infection in carriers (Satpathy *et al.*, 2026) [15]. Human transmission primarily occurs through consumption of raw or contaminated animal products, particularly unpasteurised milk products or direct contact with brucella infected animals, handling of placentas or aborted foetal materials (Dagnaw *et al.*, 2024) [3]. Furthermore, it spreads when laboratory specimens contaminated with *Brucella* spp. are handled without proper biosafety measures (Pal *et al.*, 2017) [12]. Human brucellosis can manifest as an acute, subacute or chronic febrile illness. Clinical signs include fever, headache, extreme fatigue, chills, night sweats, weight loss, joint pain and orchitis. Hepatomegaly and splenomegaly may occur in chronic cases (Bedi *et al.*, 2022) [1]. Hence, this disease is considered a socio-economically important public health concern.

Although bacterial culture is considered the gold standard for diagnosing brucellosis, the high biosafety risk of the pathogen necessitates biosafety level III, well-equipped laboratories, making cultural methods economically and practically impractical (WOAH, 2022) ^[22]. Hence, the most commonly preferred diagnostic methods are serological tests (Veccion *et al.*, 2025) ^[21]. In recent times, rapid, cost-effective, widely used serological test are buffered *Brucella* coloured antigen tests and indirect enzyme-linked immunosorbent assay (ELISA) (WOAH, 2022; Legesse *et al.*, 2023) ^[22, 9].

2. Materials and Methods

A cross-sectional study was conducted to assess the seropositivity of brucellosis in buffaloes in Coimbatore, which had a history of abortions. A total of 93 blood samples from four buffalo farms comprising farm A ($n=42$), farm B ($n=16$), farm C ($n=26$), and farm D ($n=09$), were collected in and around the Coimbatore district. All four buffalo farms had a history of third-trimester abortions. The blood samples from the animals were collected in sterile serum collection tubes and immediately transferred to the laboratory of the Department of Veterinary Public Health and Epidemiology, Veterinary College and Research Institute, Udumalpet, Tamil Nadu.

Serum was separated by centrifugation (Eppendorf, Germany) at 5000 RPM for 10 min at 15 °C. The separated serum was transferred to Cryovials and stored at – 20 °C till further use. Three assays, the *Brucella* coloured antigen plate test, the modified-coloured antigen plate test and monoclonal antibody-based blocking ELISA were used to determine the seroprevalence of *Brucella* among the buffalo farms.

2.1 Rose Bengal Plate Agglutination test (RBPT)

The Rose Bengal Plate Agglutination test (RBPT) performed as per (WOAH, 2022) ^[22]. Briefly, 30 µL of serum was placed on a white glossy ceramic tile and an equal volume of RBPT antigen was added over it. A sterile microtip was used to mix the serum and antigen. Further, the tile was gently rocked in the hand for four minutes. Results were interpreted as per Table 1.

2.2 Modified Rose Bengal Plate Agglutination test (mRBPT)

The modified Rose Bengal Plate Agglutination test was performed as per the World Organisation for Animal Health guidelines (WOAH, 2022) ^[22]. Briefly, mixing 75 µL of serum and 25 µL of the RBPT antigen. A sterile microtip was used to mix the serum and antigen. Further, the tile was gently rocked in the hand for four minutes. Results were interpreted as per Corner (1989) ^[2] given in Table 1.

2.3 Interpretation for the agglutination test

Scoring was used to interpret the results after four minutes.

Table 1: Interpretation table

Score	Degree of reaction	Interpretation
0	No agglutination and no rimming	Negative
1	Barely perceptible agglutination or some rimming	Positive
2	Fine agglutination and definite rimming	
3	Coarse clumping	

2.4 Monoclonal antibody-based blocking ELISA (c-ELISA)

The Bru alert® ELISA kit was procured from Translational Research Platform for Veterinary Biologicals (TRPVB), Chennai. The assay was performed as per the manufacturer's protocol. Briefly, all the reagents were equilibrated to room temperature (20-25°C). Prepared the template for 96 wells and marked the wells as positive control, negative control and test serum in duplicate reactions. Firstly, 90 µL of sample diluents (1X wash buffer) was added to the sample wells, and then 10 µL of the respective serum sample was added in duplicate. Then, 100 µL of positive and negative control sera to the respective control wells were added. Gently mixed the contents of the wells. Then, the plate was covered with aluminium foil and incubated for one hour at room temperature (20-25°C). Further, washed four times with 1X wash buffer (300 µL per well). Further, 100 µL of conjugate was added to each well. The plate was sealed and incubated for one hour at room temperature (20- 25°C). After incubation, washed with 1X wash buffer four times. Further, 100 µL of substrate (TMB) was added to each well and left for 10 minutes at room temperature. Finally, 100 µL of stop solution was added to each well. Five minutes after the stop solution was added, the optical density (OD) of each well was measured at 450 nm in the ELISA reader.

2.5 Cut-off analysis

Percentage Inhibition (PI) $PI=100 - [(Test\ sample\ OD / Negative\ control\ OD) \times 100]$.

Results: PI below 40 was considered negative for *Brucella* antibodies, and PI 40 and above was considered positive for *Brucella* antibodies

2.6 Statistical analysis

Descriptive statistics were used to estimate the overall and farm-wise seropositivity of brucellosis, and the results were expressed as percentages. Since every buffalo ($n = 93$) was screened simultaneously by all three assays, the tests were compared on a paired basis. According to the WOA (2022) ^[22], RBPT is highly recommended as the most suitable screening test for herd-level seroprevalence studies, while the cELISA is also recommended as a suitable assay despite certain limitations for the serosurveillance of brucellosis. Based on this recommendation, the diagnostic performance of the tests was evaluated through three pairwise comparisons: RBPT (recommended test as standard) versus modified RBPT (mRBPT), RBPT (recommended test as standard) versus cELISA and mRBPT (standard) versus cELISA. For each comparison, the test results were cross-tabulated into a 2 × 2 contingency (concordance) table (Table 2) to assess the agreement between the assays. Chance-corrected agreement between the two tests was quantified using Cohen's kappa coefficient (κ) and interpreted according to Landis and Koch (1977) ^[8].

The difference in the proportion of sero-reactors detected by the two tests was assessed with McNemar's test for paired proportions (the exact binomial form was used when discordant pairs were few, and gave concordant conclusions). The relative diagnostic performance, such as sensitivity, specificity, positive and negative predictive values (PPV, NPV) and overall accuracy of each test was estimated by

treating the designated standard test in each pair as the reference. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA) and R software Version 4.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

Table 2: 2×2 contingency table

Sensitivity (%)	$TP / (TP + FN) \times 100$
Specificity (%)	$TN / (TN + FP) \times 100$
Accuracy (%)	$(TP + TN) / N \times 100$

3. Results

Out of 93 buffalo serum samples processed, an overall seropositivity of 54.84% was observed by c-ELISA, followed by 50.54% and 26.88% by mRBPT and RBPT, respectively (Fig. 4). The distribution of seropositivity and seronegativity of the sera samples for the RBPT, mRBPT and c-ELISA is represented as a heat map in Figure 1. This study reveals that c-ELISA had better diagnostic ability than mRBPT and

RBPT. The distribution of the OD values and cut-off PI values of the buffalo serum samples in the c-ELISA is represented in Figures 2 and 3, respectively.

Farm-wise analysis revealed variation in seropositivity among the farms. In Farm A ($n = 42$), higher seropositivity was observed as 66.67% by c-ELISA and followed by 57.14% (mRBPT) and 26.19% (RBPT), with c-ELISA detecting the highest proportion of seropositive cases (Fig. 4). Similarly, in Farm B ($n = 16$), seropositivity was 56.25% by both RBPT and mRBPT, while c-ELISA showed a higher positivity of 75%, indicating a higher infectivity rate (Fig. 4).

In Farm C ($n = 26$), lower seropositivity was recorded compared to other farms, with 11.54% by RBPT, 38.46% by mRBPT and 30.77% by c-ELISA. In contrast, Farm D ($n = 9$) showed a moderate level of seropositivity with 44.44% by mRBPT and 33.33% by c-ELISA and 22.22% by RBPT (Fig. 4).

Across all farms, c-ELISA followed by mRBPT consistently detected higher seropositivity compared to the RBPT test, suggesting its greater sensitivity.

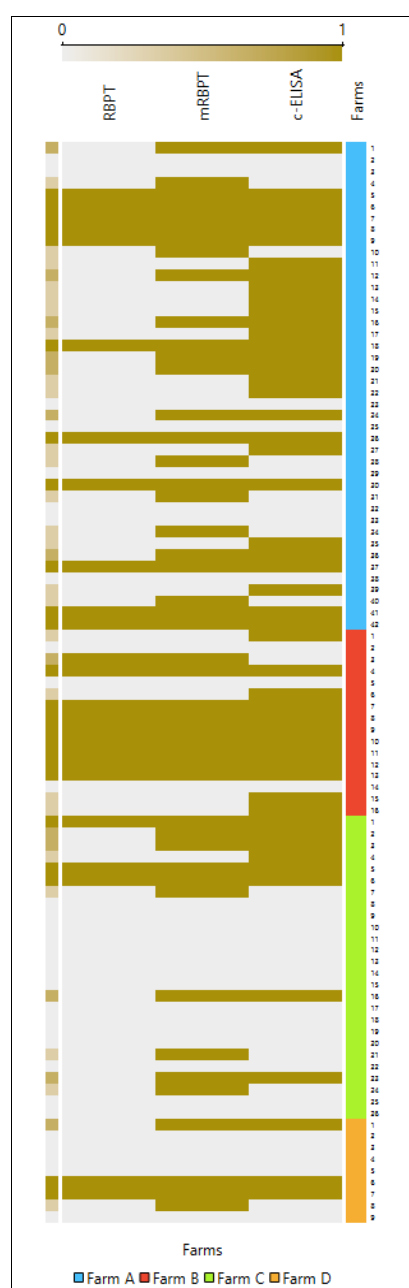


Fig 1: Heat map representing the distribution of seropositivity and seronegativity of the buffalo sera samples by the RBPT, mRBPT and cELISA. 0 - Indicates negative; 1- indicates positive

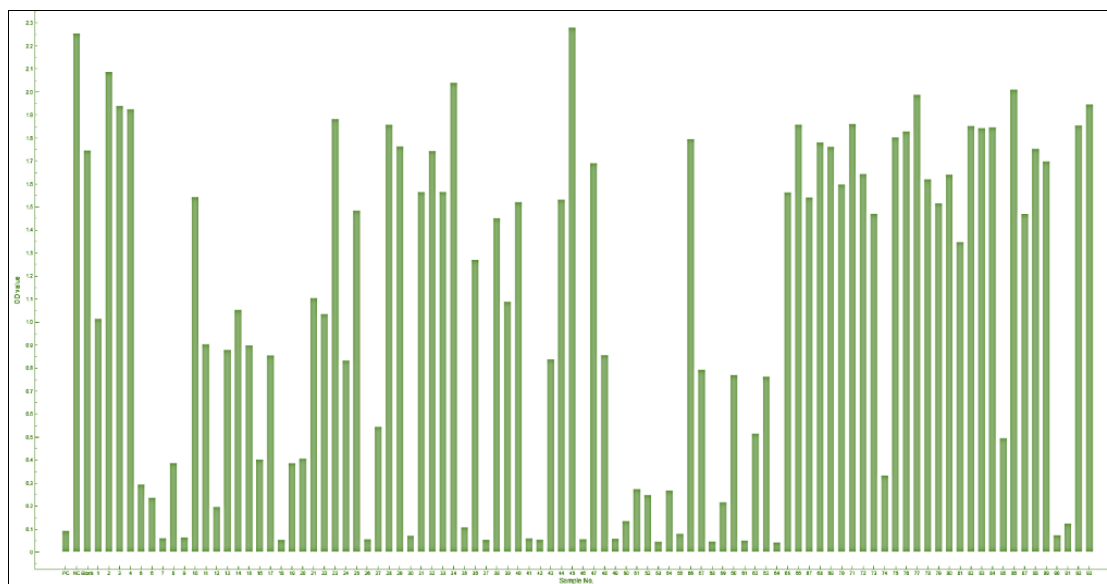


Fig 2: Distribution of average OD values of buffalo sera samples by c-ELISA. PC – positive control, NC- negative control, 1 to 93 - buffalo sera samples

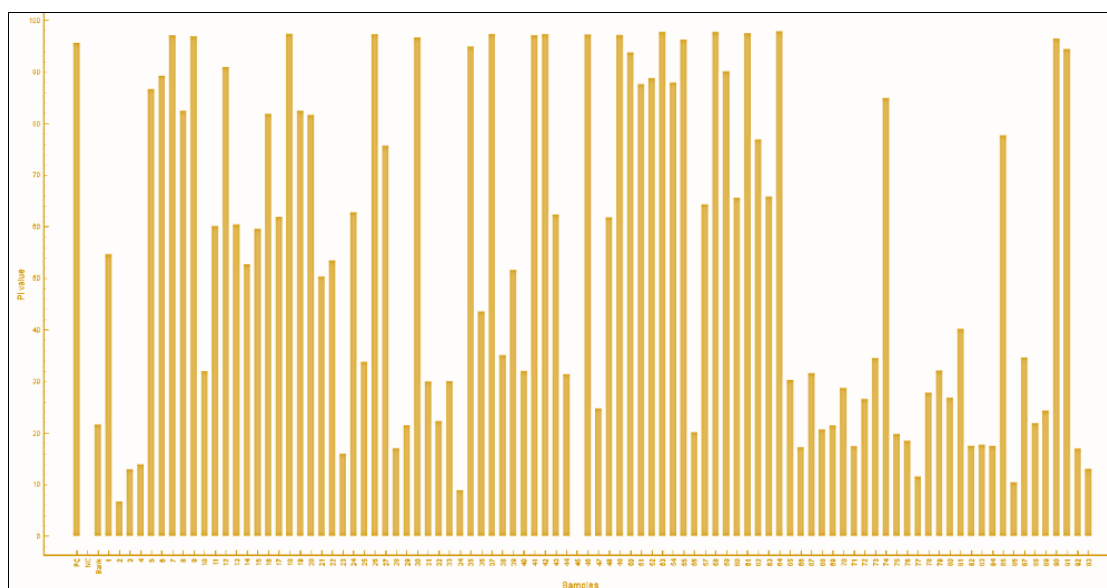


Fig 3: PI values calculated for the OD value of buffalo sera samples by c-ELISA. PC – positive control, NC- negative control, 1 to 93 - buffalo sera samples

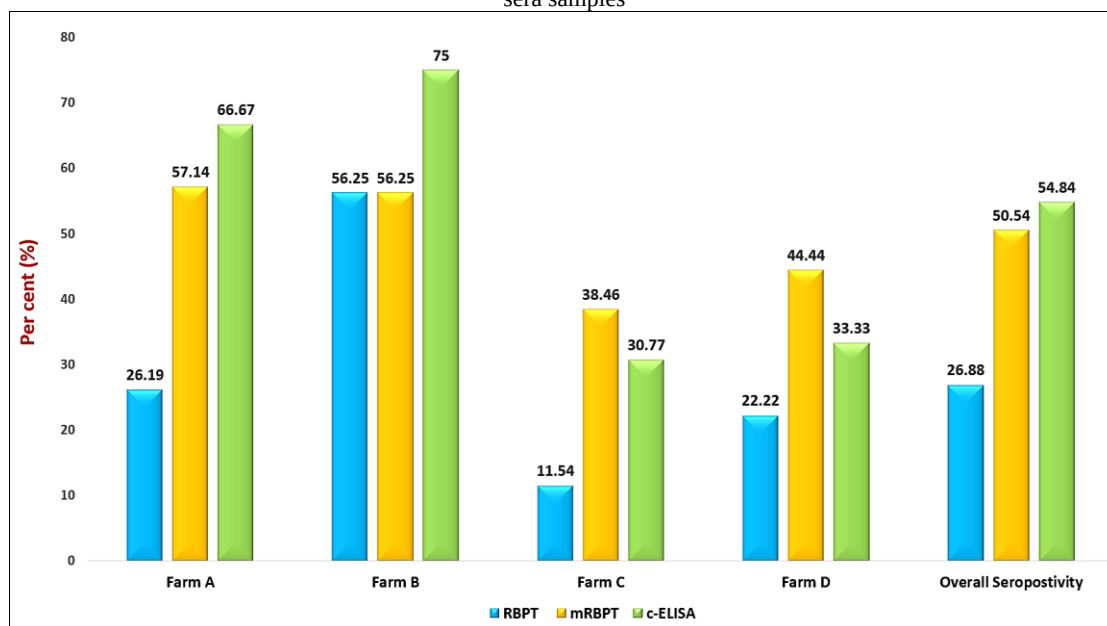


Fig 4: Seropositivity of Brucellosis among unorganised Buffalo Farms of Coimbatore district, Tamil Nadu

The agreement and relative diagnostic performance of the three serological assays were assessed using pairwise comparisons. Pairwise concordance analysis demonstrated that RBPT and mRBPT showed an overall agreement of 76.34% (Table 3), with 25 serum samples tested as positive by both assays and 46 serum samples tested as negative by both assays. However, 22 serum samples were positive only by mRBPT, while no serum sample was positive only by RBPT. The agreement between the two tests was moderate (Cohen's $\kappa = 0.529$, 95% CI: 0.357–0.701), and McNemar's test showed a statistically significant difference between the assays ($\chi^2 = 20.05$, $P < 0.0001$) (Table 4).

Comparison of RBPT and cELISA revealed an overall agreement of 69.89% (Table 3), with 24 serum samples positive and 41 serum samples negative by both tests. One sample was positive only by RBPT, whereas 27 samples were detected exclusively by cELISA. The level of agreement was also moderate ($\kappa = 0.424$, 95% CI: 0.245–0.602), with a significant McNemar's test result ($\chi^2 = 22.32$, $P < 0.0001$), suggesting that cELISA identified significantly more seropositive animals than RBPT (Table 4).

The comparison between mRBPT and cELISA showed an overall agreement of 72.04% (Table 3), with 36 samples positive and 31 samples negative by both assays. Eleven

samples were positive only by mRBPT, while 15 were positive only by cELISA. The agreement between these assays was moderate ($\kappa = 0.440$, 95% CI: 0.258–0.623). In contrast to the previous comparisons, McNemar's test was not statistically significant ($\chi^2 = 0.35$, $P = 0.556$), indicating comparable detection rates between mRBPT and cELISA (Table 4).

The relative diagnostic performance of the assays was further assessed using pairwise comparisons (Table 4). When RBPT was considered the reference standard, mRBPT demonstrated a sensitivity of 100.00%, specificity of 67.65%, positive predictive value (PPV) of 53.19%, negative predictive value (NPV) of 100.00%, and an overall accuracy of 76.34%. Similarly, cELISA exhibited a sensitivity of 96.00%, specificity of 60.29%, PPV of 47.06%, NPV of 97.62%, and an accuracy of 69.89% relative to RBPT.

Using mRBPT as the reference standard, cELISA achieved a sensitivity of 76.60%, specificity of 67.39%, PPV of 70.59%, NPV of 73.81%, and an overall accuracy of 72.04%. These findings indicate that mRBPT detected all RBPT-positive animals while identifying additional seroreactive animals, and its diagnostic performance was broadly comparable with cELISA.

Table 3: Relative diagnostic performance of each test against its paired standard (n = 93)

Comparison (Test vs Standard)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
mRBPT vs RBPT (standard)	100.00	67.65	53.19	100.00	76.34
cELISA vs RBPT (standard)	96.00	60.29	47.06	97.62	69.89
cELISA vs mRBPT (standard)	76.60	67.39	70.59	73.81	72.04

PPV = positive predictive value; NPV = negative predictive value. Values are computed relative to the designated standard test in each pair.

Table 4: Pairwise concordance, agreement and McNemar's test for the three serological assays (n = 93)

Comparison (Standard vs Test)	Both reactive (+/+)	Standard reactive only	Test reactive only	Both non-reactive (-/-)	Agreement (%)	Cohen's κ (95% CI)	McNemar χ^2 (P)
RBPT (Standard) vs mRBPT	25	0	22	46	76.34	0.529 (0.357–0.701)	20.05 (< 0.0001)
RBPT (Standard) vs cELISA	24	1	27	41	69.89	0.424 (0.245–0.602)	22.32 (< 0.0001)
mRBPT (Standard) vs cELISA	36	11	15	31	72.04	0.440 (0.258–0.623)	0.35 (0.556)

Standard reactive only = standard test positive but comparator negative; Test reactive only = comparator positive but standard negative. Strength of agreement (Landis & Koch): κ 0.41–0.60 = moderate.

4. Discussion

In the current study, overall brucellosis seropositivity was 54.84% by c-ELISA, followed by 50.54% and 26.88% by mRBPT and RBPT, respectively among the unorganised buffalo farms of Coimbatore, Tamil Nadu had a history of abortions. This observation suggests that the c-ELISA had a better diagnostic ability. Results from the current study were comparable to the findings of Tluway *et al.*, (2025) [19], who revealed a brucellosis seroprevalence of 51.9% from cattle farms in Tanzania using RBPT and c-ELISA. The Hussain *et al.*, (2025) [5] also observed a seropositivity of 54.8% among buffaloes in Pakistan using the indirect ELISA (i-ELISA). In contrast, purposively evaluated samples from buffalo farms with an abortion history showed a relatively high seropositivity of 72%, whereas animals without an abortion history had a seroprevalence of 13.04% and were reported in Gujarat (Trangadia and Patel 2016) [20]. Using c-ELISA, 90% seroprevalence was recorded in Patiala buffaloes (Shome *et al.*, 2024) [16].

Shome *et al.*, (2024) [16] conducted a snapshot study on brucellosis seropositivity among buffaloes all over India using c-ELISA and reported that seropositivity for brucellosis in Punjab state as 57%, followed by Andhra Pradesh, Tamil Nadu, Haryana, Maharashtra and Uttar Pradesh as 28%, 19%,

15.6%, 13.89% and 12.87%, respectively. The lowest seropositivity among buffaloes of Karnataka, Gujarat and Madhya Pradesh with 8.82%, 5.13%, 0.9%, respectively. In contrast, Yuguda *et al.*, (2019) [23] observed lower seropositivity of 6.66% and 5% among buffaloes by RBPT and c-ELISA, respectively in Chennai, Tamil Nadu. Mittal *et al.*, (2025) [11] also observed 4.37% among the buffaloes at Haryana using RBPT and i-ELISA.

When RBPT was compared with mRBPT, tests agreed on 71 of 93 buffaloes (76.34%), giving moderate chance-corrected agreement ($\kappa = 0.529$). Notably, every RBPT-reactive animal was also reactive by mRBPT, while mRBPT detected 22 additional reactors that RBPT missed, raising the apparent seroprevalence from 26.88% to 50.54%. This one-directional disagreement was highly significant on McNemar's test ($\chi^2 = 20.05$; $P < 0.0001$), confirming that mRBPT detects significantly more sero-reactors than the conventional RBPT. The mRBPT showed 100% relative sensitivity and 67.65% relative specificity.

When RBPT was compared with cELISA, agreement was 69.89% (65/93) with moderate concordance ($\kappa = 0.424$). The cELISA classified 51 buffaloes (54.84%) as reactive against 25 (26.88%) by RBPT, with 27 RBPT-negative/cELISA-positive animals and only one RBPT-positive/cELISA-

negative animal. McNemar's test was highly significant ($\chi^2 = 22.32$; $P < 0.0001$), showing that cELISA detects significantly more reactors than RBPT. Relative to RBPT, cELISA had 96.00% sensitivity and 60.29% specificity; the high NPV (97.62%) alongside a low PPV (47.06%) reflects RBPT's tendency to miss true reactors that cELISA identifies.

When mRBPT was compared with cELISA, agreement was 72.04% (67/93) with moderate concordance ($\kappa = 0.440$). The proportion of reactors was similar for the two tests (mRBPT 50.54% versus cELISA 54.84%) and the discordances were balanced (11 mRBPT-positive/cELISA-negative versus 15 mRBPT-negative/cELISA-positive). McNemar's test was not significant ($\chi^2 = 0.35$; $P = 0.556$), indicating no systematic difference in the number of reactors detected by mRBPT and cELISA. Relative to mRBPT, cELISA showed 76.60% sensitivity and 67.39% specificity.

Similar findings have been reported by previous workers, where field-based serological tests such as RBPT and mRBPT demonstrated moderate concordance with c-ELISA due to variations in antibody titres, stage of infection, and cross-reacting antibodies. Matope *et al.* (2011) ^[10] observed that RBPT showed less sensitivity than c-ELISA, although both the screening tests were useful for herd-level brucellosis identification. Similarly, Sarangi *et al.* (2022) ^[14] reported variability in the diagnostic sensitivity of the serological assays used to detect brucellosis in cattle and buffaloes during an endemic situation. In contrast to the current study findings, several authors have reported considerably higher specificity and sensitivity for c-ELISA and related serological assays. Matope *et al.* (2011) ^[10] observed specificity higher than 95% for the c-ELISA, while other studies demonstrated sensitivity of the diagnostic test ranging from 98 to 99% under controlled epidemiological conditions. These differences may be due to variations in geographical prevalence, sample size, vaccination status, management practices, and the epidemiological status of the study population.

5. Conclusion

Brucellosis poses a significant challenge affecting both humans and animals around the world, with considerable public health and economic impact. In India, bovine brucellosis is considered a zoonotic disease that causes economic losses due to its widespread prevalence and its involvement with reproductive disorders, particularly abortion among livestock. The findings of this study suggested that the mRBPT exhibited the highest sensitivity relative to RBPT and detected additional seropositive animals without compromising its negative predictive value. In field conditions, where appropriate facilities for performing c-ELISA are not available, the mRBPT approach may be utilised as a quick, practical point-of-care screening assay. Whereas c-ELISA showed diagnostic performance comparable to mRBPT, supporting its role as a reliable confirmatory assay in brucellosis surveillance among buffaloes reared in unorganised farming systems.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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