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Evaluation of serological assays for the detection of bovine brucellosis and optimization of an in-house rose Bengal antigen

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Abstract

Rapid and accurate diagnosis is vital for the control of bovine brucellosis. This study evaluated the diagnostic performance of serological assays, including the Rose Bengal Test (RBT), Standard Tube Agglutination Test (STAT), indirect Enzyme-Linked Immunosorbent Assay (i-ELISA), and Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA), across 236 bovine serum samples. Additionally, an in-house RBT antigen utilizing *Brucella abortus* S99 was successfully optimized (pH 3.65, 8% PCV), demonstrating performance identical to commercial kits. Overall seropositivity rates were 51.1% (RBT), 41.9% (STAT), 52.5% (i-ELISA), and 58.4% for c-ELISA. Using c-ELISA as the relative reference standard, i-ELISA achieved the highest sensitivity (87.05%) and specificity (96.91%), while STAT showed high absolute specificity but the lowest sensitivity (69.01%). The findings indicate that while the optimized in-house RBT provides reliable, cost-effective field screening, traditional agglutination tests lack the standalone sensitivity required for chronic cases. Consequently, highly accurate ELISA-based methods are recommended as essential confirmatory assays for robust brucellosis surveillance.

Keywords: Bovine brucellosis, serological assays, Rose Bengal Test (RBT)

1. Introduction

Brucellosis is widely regarded as one of the world's most important zoonotic diseases, affecting livestock productivity and human health. In India, it remains highly endemic, with reported prevalence rates in livestock ranging from 6.5% to 16.4% [1, 2, 3, 4, 5]. The disease in animals is primarily characterized by reproductive disorders, including abortions and stillbirths [6]. The serological diagnosis of bovine brucellosis is usually performed using a stepwise approach that combines screening with confirmatory assays. Bacterial culture is the gold standard but is time-consuming and requires high-biosafety laboratory conditions. Consequently, serodiagnosis remains the cornerstone of surveillance. Commonly used methods include the Rose Bengal test (RBT), standard tube agglutination test (STAT), and indirect enzyme-linked immunosorbent assay (i-ELISA) [7, 8]. This study aimed to develop and optimize a cost-effective in-house RBT antigen and comprehensively evaluate its performance alongside established conventional and ELISA-based serological assays to improve disease surveillance in bovine herds.

2. Materials and Methods

2.1. Specimens: A total of 236 blood samples (Table 2.1) were collected from cattle with a history of abortion from farms in the Pune and Mumbai regions. Serum was separated and stored at -20°C for serological testing. Standard reference strains (*B. melitensis* 16M, Rev1, *B. abortus* S99, S19, 544) were acquired from IVRI, Izatnagar.

Table 2.1: Details of the cattle samples with a history of abortion collected for investigation

Sr. No.	Name of the farm	Type of sample collected	Region	Sample code	No. of sample(s)
1.	Farm 1	Blood	Pune	S1-S132	132
2.	Farm 2	Blood	Mumbai	S133-S161	29
3.	Farm 3	Blood	Pune	S162-S236	75
Total samples collected					236

2.2. Development of In-house Rapid Assay (RBT): The reference strain *Brucella abortus* S99 was maintained on *Brucella* Agar Medium, harvested using phenol saline, and inactivated at 65°C for 60 min. Cells were stained with 1% Rose Bengal dye and suspended in a buffered diluent. The final packed cell volume (PCV) was adjusted to 8%, and the pH was standardized to 3.65 ± 0.05 [9].

2.3. Serological Techniques:

- **RBPT:** Serum was tested using both in-house and commercial antigens. Equal volumes (25-30 µl) of serum and antigen were mixed and observed for agglutination after 4 min.
- **STAT:** Serum was serially diluted in carbol saline, mixed with *B. abortus* antigen, and incubated at 37°C for 24h. Titers >80 IU/ml were considered positive.
- **ELISA:** Commercial i-ELISA and c-ELISA kits were used according to the manufacturer's instructions. A commercially available *Brucella* antibody detection diagnostic kit and antigen were obtained from Texas (USA) and Uttar Pradesh (India). The *Brucella*-Ab C-ELISA kit was obtained from INDICAL BIOSCIENCE GmbH, (Leipzig, Germany). For c-ELISA, the percent inhibition (PI) of the samples was calculated, with values

> 30% considered positive.

2.4. Statistical Analysis: Diagnostic parameters (sensitivity, specificity, PPV, and NPV) were calculated using c-ELISA as the reference test (Table 2.2). Agreement was evaluated using Cohen's kappa coefficient.

Table 2.2: Recording and analysis of data for determining sensitivity, specificity and overall agreement of tests

		Gold standard test		Total
		Positive	Negative	
The test to be compared	Positive	a	b	a+b
	Negative	c	d	c+d
Total		a+c	b+d	a+b+c+d=N

3. Results

3.1. Serological Detection: Out of 236 serum samples, both the commercial and in-house RBT antigens yielded identical results, detecting 121 positive samples (51.1%). The STAT yielded a lower overall prevalence, detecting 99 positive samples (41.9%). ELISA-methods demonstrated higher detection rates: i-ELISA detected 124 positives (52.54%), and c-ELISA identified 138 positives (58.4%) [Table 3.1, 3.2, 3.3 and 3.4]

Table 3.1: Detection of *Brucella* antibodies in bovine sera by RBT (In-house and Commercial antigen)

Sr. No.	Source of serum samples	No. of samples tested	No. of samples positive	Percentage of positivity
1.	Farm 1	132	80	60.6%
2.	Farm 2	29	13	44.8%
3.	Farm 3	75	28	37.3%
Total		236	121	51.1%

Table 3.2: Detection of *Brucella* antibodies in bovine sera by STAT

Sr. No.	Source of serum samples	No. of samples tested	No. of samples positive	Percentage of positivity
1.	Farm 1	132	67	50.7%
2.	Farm 2	29	08	27.5%
3.	Farm 3	75	24	32%
Total		236	99	41.9%

Table 3.3: Detection of *Brucella* antibodies in bovine sera by i-Elisa

Sr. No.	Source of serum samples	No. of samples tested	No. of samples positive	Percentage of positivity
1.	Farm 1	132	80	60.6%
2.	Farm 2	29	13	44.8%
3.	Farm 3	75	31	41.3%
Total		236	124	52.54%

Table 3.4: Detection of *Brucella* antibodies in bovine sera by C-Elisa

Sr. No.	Source of serum samples	No. of samples tested	No. of samples positive	Percentage of positivity
1.	Farm 1	132	85	64.3%
2.	Farm 2	29	13	44.8%
3.	Farm 3	75	40	53.3%
Total		236	138	58.4%

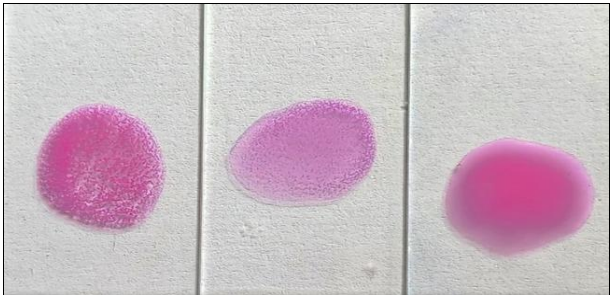


Fig 3.1: Detection of *Brucella* antibodies by Commercial and In-house RBT antigen

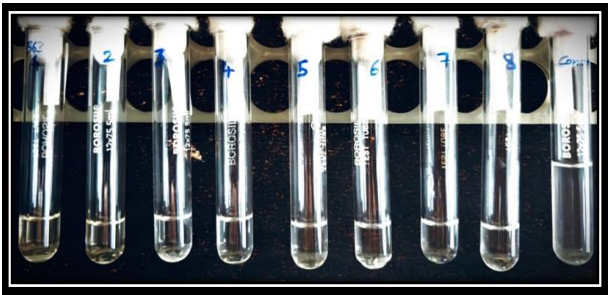


Fig 3.2: Detection of *Brucella* antibodies by Standard tube agglutination test

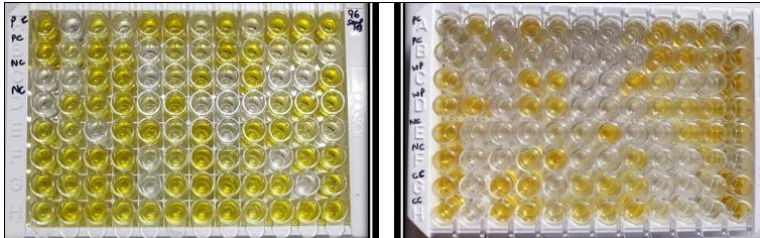


Fig 3.3: Detection of *Brucella* antibodies by i-ELISA and C-ELISA

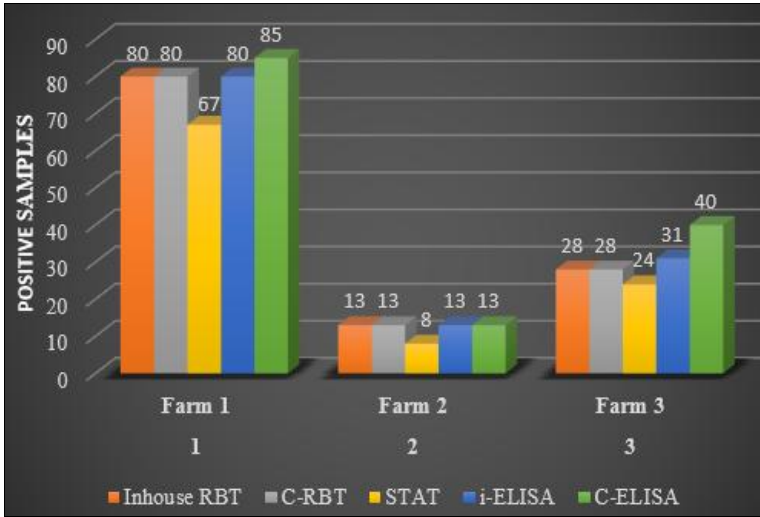


Fig 3.4: Farm wise seroprevalence of *Brucella* antibodies in serum samples detected by various serological assays

3.2. Comparative Evaluation: Using c-ELISA as the reference, i-ELISA demonstrated the highest sensitivity (87.05%) and specificity (96.91%) with almost perfect agreement ($\kappa = 0.820$). The RBT demonstrated moderate

sensitivity (74.29%) and specificity (82.29%). STAT exhibited the lowest sensitivity (69.01%) but the highest absolute specificity (98.94%) (Table 3.5)

Table 3.5: Efficacy of different serological assays compared to c-ELISA

Serological & Molecular tests	C-ELISA and In-house RBT	C-ELISA and Commercial RBT	C-ELISA and STAT	C-ELISA and i-Elisa	C-ELISA and PCR
Sensitivity	74.29%	74.29%	69.01%	87.05%	96.50%
Specificity	82.29%	82.29%	98.94%	96.91%	83.87%
Overall agreement	77.54%	77.54%	80.93%	91.10%	91.53%
PPV	85.95%	85.95%	98.99%	97.58%	90.20%
NPV	68.70%	68.70%	67.88%	83.93%	93.98%
% False Positive	14.05%	14.05%	1.01%	3.09%	16.13%
% False Negative	31.30%	31.30%	32.12%	12.95%	3.50%
K value	0.549	0.549	0.631	0.820	0.819
Strength of agreement	Moderate agreement	Moderate agreement	Good agreement	Very good agreement	Good agreement
P value	0.0127	0.0127	< 0.0001	0.0015	0.0414

4. Discussion

The Rose Bengal Plate Test (RBPT) is widely used as a primary rapid screening tool due to its simplicity [11]. Our optimized in-house RBT antigen demonstrated identical

diagnostic performance to commercial kits. While some studies report higher positivity with local antigens due to circulating strain differences [12, 13, 14]. Our findings confirm comparable efficacy between standardized in-house and

commercial preparations ^[15]. However, an elevated false-negative rate (31.30%) in RBT indicates chronic cases may be missed if RBT is used in isolation. STAT primarily detects early agglutinating antibodies (IgM and IgG) ^[16], achieving exceptionally high specificity (98.94%). However, its limited sensitivity (69.01%) reflects its inability to detect non-agglutinating antibodies dominating chronic stages ^[17, 18, 19]. ELISA-based methods overcome these limitations, demonstrating superior capacity to detect both antibody types, yielding a high sensitivity (87.05%) and perfect agreement with the c-ELISA reference ^[20, 21, 2].

5. Conclusion

An in-house RBT antigen was successfully optimized from *B. abortus* S99, demonstrating field-level performance comparable to commercial antigens. While conventional tests like RBT and STAT remain practical and cost-effective for screening, their diagnostic sensitivity is inherently limited during chronic infection. Therefore, combining these rapid screening tools with highly sensitive ELISA methods is recommended to ensure robust case detection in bovine brucellosis control programs.

Declarations

Data Availability Statement: The raw data supporting the conclusions of this study will be made available by the authors upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Biosafety Committee (IBSC) of Mumbai Veterinary College (Approval number: MUMB071220222542 / Meeting No. 4 / S. No 5 / Agenda Item No. 1.5 / Date: 29-07-2025).

Author Contributions

K. J. Reddy: Investigation, Methodology, Data curation, Formal analysis, and Writing – original draft.

R. R. Pharande: Conceptualization, Supervision, Methodology and Writing – review and editing.

R. S. Gandge: Validation, Methodology, and Writing – review and editing.

V. D. Thorat: Resources, Data curation, and molecular assay support.

S. D. Ingole: Formal analysis and experimental validation.

R. J. Zende: Epidemiological investigation, Resources, and Writing – review and editing.

All authors have read and agreed to the published version of the manuscript.

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