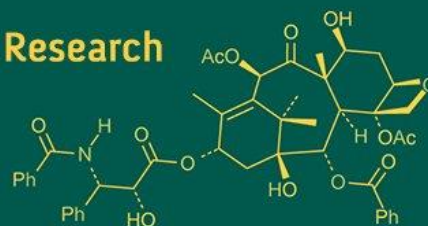
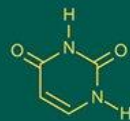


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Prevalence of bovine brucellosis and molecular characterization of *Brucella abortus* virulence genes (*omp19* and *ure*) in western India

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Abstract

Bovine brucellosis caused by *Brucella abortus* remains an endemic zoonosis worldwide, producing substantial economic losses through reproductive failure in dairy cattle and significant public health risks. This study evaluates the prevalence of brucellosis in cattle presenting with a history of abortion from the Pune and Mumbai regions of Maharashtra, India, and characterizes circulating strains using serological, molecular, and phylogenetic approaches. A total of 400 bovine clinical samples (200 milk and 200 blood/serum) were screened using the Milk Ring Test (MRT) and Rose Bengal Plate Test (RBPT). Genus- and virulence-specific conventional Polymerase Chain Reaction (PCR) assays were performed targeting the *BCSP31* (223 bp), *omp19* (550 bp), and *ure* (2100 bp) genes. Overall seropositivity was 54.0% (108/200) by MRT and 27.5% (55/200) by RBPT, with consistently higher positivity rates in Pune compared to Mumbai. Molecular screening of 163 seropositive clinical specimens using *BCSP31* PCR revealed an overall detection rate of 72.39% (118/163; 78.9% in Pune milk, 66.7% in Pune blood, 61.5% in Mumbai milk, and 50.0% in Mumbai blood). Amplification of virulence markers revealed 27.6% (45/163) positivity for *omp19* and 33.7% (55/163) for the urease (*ure*) gene. Partial gene sequencing and phylogenetic analysis demonstrated high sequence identity of three field-derived isolates (Samples 1, 3, and 4) with the *B. abortus* S19 reference vaccine strain, whereas Sample 2 clustered distinctly with wild-type field strains. These findings highlight significant endemic exposure, active milk shedding of *Brucella*, and the coexistence of field and vaccine-related lineages within dairy herds, underlining the necessity for integrated molecular diagnostics and genomic surveillance in regional control programs.

Keywords: Bovine brucellosis, *Brucella abortus*, *BCSP31*, *OMP19*, *ure* gene, milk ring test, rose Bengal plate test, molecular epidemiology

Introduction

Brucellosis is a globally distributed zoonotic infection affecting domestic livestock and humans (Seleem *et al.*, 2010; Khairullah *et al.*, 2024). In cattle, the disease is primarily caused by *Brucella abortus*, an intracellular bacterium characterized by tropism for the reproductive tract (Barbuddhe *et al.*, 2024). Clinical manifestations include late-gestation abortions, placentitis, retained fetal membranes, stillbirths, and infertility, resulting in reduced milk yield and severe financial distress to livestock producers (Leishangthem *et al.*, 2019; Awais *et al.*, 2024). Humans acquire the infection primarily via ingestion of unpasteurized milk and raw dairy products or through occupational contact with infected tissues, leading to debilitating undulant fever, arthritis, and chronic systemic complications (Kumar *et al.*, 1997; Islam *et al.*, 2023) [55, 27, 15, 33, 13, 29, 25].

Routine surveillance in livestock has traditionally relied on conventional serological methods

- **Rose Bengal Plate Test (RBPT):** A rapid, cost-effective agglutination test suitable for herd-level serum screening (Khan *et al.*, 2017; Alamian *et al.*, 2023).
- **Milk Ring Test (MRT):** A non-invasive method targeting secretory immunoglobulin-antigen complexes in bulk or individual milk supplies (Mohamand *et al.*, 2014; Shome *et al.*, 2015) [28, 5, 40, 56].

Serological assays can be compromised by cross-reactions with other Gram-negative organisms (such as *Yersinia enterocolitica* O:9) and an inability to distinguish active bacteremia from post-vaccinal antibody titers (Asefa and Fesseha, 2022). Consequently, nucleic acid amplification techniques targeting conserved loci most notably the 31-kDa *Brucella* cell surface protein gene (*BCSP31*) provide higher diagnostic sensitivity and specificity directly from clinical samples without the biosafety risks associated with live culture isolation (Baily *et al.*, 1992; Vaid *et al.*, 2004; Satei *et al.*, 2020).

Beyond genus-level confirmation, determining the pathogenic profile of circulating strains requires evaluation of specific virulence factors:

Outer Membrane Protein 19 (*omp19*): A 19-kDa surface lipoprotein critical for maintaining outer membrane integrity, surviving host microbicidal systems, and mediating immune evasion (Mirnejad *et al.*, 2017; Paladan, 2023).

Urease Operon (*ure*): Enables acid resistance during gastric transit and survival inside host macrophages by hydrolyzing urea into ammonia and carbon dioxide (Esmacel, 2019; Papaparaskevas *et al.*, 2023) [11, 14, 63, 54, 39, 43, 20, 44].

This study investigates the prevalence of brucellosis across commercial dairy herds in Pune and Mumbai (Maharashtra, India) using MRT and RBPT, evaluates direct molecular detection via *BCSP31* PCR, analyzes the distribution of *omp19* and *ure* virulence markers, and determines the phylogenetic relatedness of local isolates with field and vaccine (*B. abortus* S19) lineages.

Materials and Methods

Study Design and Sample Collection

A total of 400 clinical specimens were gathered from dairy cattle with a verified history of recent abortion housed across two facilities in Maharashtra, India: Bhagyaxmi Dairy Farm (Pune; n=303) and Nandanavan Gaushala (Mumbai; n=97).

Milk Samples (n = 200): Pune (n = 170), Mumbai (n = 30). Midstream milk (8-10 mL) was collected aseptically from all four quarters after teat disinfection and discarding the first three streams

Blood and Serum (n = 200): Pune (n = 133), Mumbai (n = 67). Paired blood collections via jugular venipuncture were partitioned into plain vacutainers (incubated at ambient

temperature for 2 h to harvest serum for RBPT) and sterile EDTA vacutainers (stored at -20°C for direct genomic DNA extraction).

Serological Screening

Milk Ring Test (MRT): Conducted according to standardized guidelines (Shome *et al.*, 2015) using haematoxylin-stained antigen (ICAR-IVRI, Izatnagar). Briefly, 50 µL of antigen was mixed with 1 mL of whole milk (25 mm column height) and incubated at 37°C for 1 h. Formation of a dark pink/blue cream ring above a cleared white milk column was scored positive.

Rose Bengal Plate Test (RBPT): Performed by mixing equal 30 µL aliquots of bovine serum and RBPT-colored antigen (ICAR-IVRI) on a clean glass plate (Gürbilek *et al.*, 2017). Macroscopic agglutination within 4 min indicated positivity.

Genomic DNA Extraction

From Milk: Carried out using the NET/SDS/Proteinase K protocol with phenol:chloroform:isoamyl alcohol (25:24:1) extraction and isopropanol precipitation in the presence of 3 M sodium acetate (pH 5.5) (Dubey *et al.*, 2017)

From Whole Blood: Extracted following RBC lysis (17 mM Tris-HCl, 140 mM NH₄Cl), leukocyte lysis (10 mM Tris-HCl, 100 mM NaCl, 1% SDS, 2% Triton X-100), and Proteinase K digestion, followed by organic extraction and 70% ethanol washes (Ingle *et al.*, 2023).

Reference Strain

Brucella abortus S19 (Division of Biological Standardization, ICAR-IVRI) DNA was extracted using lysozyme-CTAB-proteinase K lysis as a positive control (Thorat, 2019) [56, 23, 19, 24, 60, 57, 41, 60].

DNA purity and concentrations were confirmed spectrophotometrically (A_{260}/A_{280} ratio using Epoch BioTek) and resolved on 0.8% agarose gels in 1× TAE buffer.

PCR Amplification of Diagnostic and Virulence Markers

Polymerase chain reactions were prepared in 25 µL reaction mixtures containing: 1× PCR buffer with MgCl₂ (4.5 µL), 0.2 mM dNTPs (0.5 µL), 10 pmol of forward and reverse primers (1.0 µL each), 0.2 µL Taq DNA polymerase, 200 ng template DNA (1.0 µL), and 16.8 µL nuclease-free water.

Table 1: Primer Sequences and Thermal Cycling Protocols

Target Gene	Primer Name	Sequence (5' → 3')	Product Size (bp)	Thermal Cycling Profile
BCSP31	B4-F B5-R	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC	223	95°C for 3 min; 35 cycles of (95°C for 30 s, 63°C for 45 s, 72°C for 45 s); final extension at 72°C for 10 min.
omp19	OMP-19-F OMP-19-R	TGA-TGG-GAA-TTT-CAA-AAG-CA GTT-TCC-GGG-TCA-GAT-CAG-C	550	95°C for 4 min; 32 cycles of (95°C for 30 s, 58°C for 45 s, 72°C for 60 s); final extension at 72°C for 7 min.
ure	Ure-F Ure-R	GCT-TGC-CCT-TGA-ATT-CCT-TTG-TGG ATC-TGC-GAA-TTT-GCC-GGA-CTC-TAT	2100	95°C for 4 min; 32 cycles of (95°C for 30 s, 61°C for 45 s, 72°C for 60 s); final extension at 72°C for 7 min.

Amplicons were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide (0.5 µg/mL) and visualized under a Bio-Rad Gel Doc EZ imaging system.

Sequencing and Phylogenetic Reconstruction

Four representative PCR products were sequenced bidirectionally (Eurofins Genomics India Pvt. Ltd., Bengaluru). Chromatograms were curated using Chromas

Lite v2.01, aligned against GenBank accessions via NCBI BLASTn, and analyzed using the Maximum Likelihood (ML) method under the Tamura-Nei model in MEGA11 software with 1,000 bootstrap replicates (Tamura *et al.*, 2021; Motegaonkar *et al.*, 2024) ^[41].

Results

Seroprevalence via MRT and RBPT

Serological testing revealed widespread exposure to *Brucella* among aborted animals:

MRT: 108 of 200 raw milk samples showed positive ring formation, representing an overall seroprevalence of 54.0%. Regionally, Pune demonstrated higher positivity (55.8%, 95/170) compared to Mumbai (43.3%, 13/30).

RBPT: 55 of 200 serum samples exhibited agglutination, yielding an overall seroprevalence of 27.5%. Positivity was notably elevated in Pune (38.83%, 45/133) compared to Mumbai (14.92%, 10/67).

Table 2: Serological prevalence of bovine brucellosis across study regions

Diagnostic Test	Region	Total Samples Tested	Positive Samples (n)	Positivity (%)
Milk Ring Test (MRT)	Pune	170	95	55.8%
	Mumbai	30	13	43.3%
	Total	200	108	54.0%
Rose Bengal Test (RBPT)	Pune	133	45	38.83%
	Mumbai	67	10	14.92%
	Total	200	55	27.5%

Molecular Detection via *BCSP31* Genus-Specific PCR

Out of the 163 seropositive clinical specimens tested (108 milk and 55 blood), *BCSP31* PCR generated the expected 223 bp amplicon in 118 samples (72.39% overall positivity).

Detection was markedly higher in milk specimens (76.85%, 83/108) compared to blood specimens (63.63%, 35/55). The Pune cohort showed higher overall molecular positivity (75.0%, 105/140) than the Mumbai cohort (56.55%, 13/23).

Table 3: Molecular confirmation of *Brucella* spp. by *BCSP31* PCR (223 bp)

Region	Sample Matrix	Samples Tested (n)	PCR Positive (n)	Positivity (%)	Region
Pune	Milk	95	75	78.9%	Pune
	Blood	45	30	66.66%	
Mumbai	Milk	13	8	61.5%	Mumbai
	Blood	10	5	50.0%	

Table 4: Diagnostic Positivity Comparison across 163 Screened Animals

Diagnostic Assay / Target	Target Matrix / Sample Type	Total Screened (N)	Positive Samples (n)	Positivity (%)
Milk Ring Test (MRT)	Raw Milk (Herd / Individual Screening)	200	108	54.0%
Rose Bengal Plate Test (RBPT)	Serum (Serological Screening)	200	55	27.5%
<i>BCSP31</i> PCR	Blood & Milk (Direct Molecular Detection)	163	118	72.39%
Urease Gene (<i>ure</i> PCR)	Blood & Milk (Virulence Characterization)	163	55	33.7%
Outer Membrane Protein (<i>omp19</i> PCR)	Blood & Milk (Virulence Characterization)	163	45	27.6%

Virulence Gene Profiles (*omp19* and *ure*)

***omp19* Gene (550 bp):** Amplified in 45 of 163 samples (27.6%). Detection was distributed across 35 milk samples (77.7% of positives) and 10 blood samples (22.2% of positives). Regional positivity was 27.8% (39/140) in Pune and 26.0% (6/23) in Mumbai.

Urease Gene (*ure*, 2100 bp): Amplified in 55 of 163 samples (33.7%), comprising 43 milk and 12 blood specimens. Positivity reached 33.57% (47/140) in Pune and 34.78% (8/23) in Mumbai.

Table 5: Distribution of *omp19* and *ure* virulence genes in clinical specimens

Target Gene	Region	Sample Type	Sample Size	Positive (n)	Positivity (%)
<i>omp19</i> (550 bp)	Pune	Milk	95	31	32.6%
		Blood	45	8	17.1%
	Mumbai	Milk	13	4	30.7%
		Blood	10	2	20.0%
	Total		163	45	27.6%
<i>ure</i> (2100 bp)	Pune	Milk	95	38	40.0%
		Blood	45	9	20.0%
	Mumbai	Milk	13	5	38.0%
		Blood	10	3	30.0%
	Total		163	55	33.7%

Sequence Homology and Phylogenetic Relatedness: BLASTn queries confirmed that all sequenced amplicons

exhibited high nucleotide homology (87% to 96%) with authenticated *Brucella* entries in GenBank.

Table 6: Sequence identity and GenBank matches of sequenced PCR products

Study Sample	Sequence Identity (%)	Assigned Genus/Species	Top NCBI Accession Alignments
Sample 1	89%	<i>Brucella</i> spp.	AM040264.1, OY741352.1, CP081455.1
Sample 2	88%	<i>Brucella</i> spp.	CP081381.1, CP007738.1, CP098097.1
Sample 3	96%	<i>Brucella</i> spp.	CP081441.1, LT671512.1, CP081453.1
Sample 4	87%	<i>Brucella</i> spp.	CP023211.1, CP098097.1, CP023211.1

Phylogenetic reconstruction segregated the isolates into two distinct evolutionary clades:

Vaccine-Associated Lineage: Samples 1, 3, and 4 clustered tightly with the reference *Brucella abortus* S19 strain with short branch lengths, indicating high conservation and potential persistence or ancestral sharing with the S19 vaccine strain (Thorat, 2019; Motegaonkar *et al.*, 2024)^[60, 41].

Wild-Type Field Lineage: Sample 2 grouped separately with virulent wild-type reference strains (*B. abortus* BFY / 106598 / 28375), highlighting genuine natural circulation of pathogenic field lineages (Wattam *et al.*, 2014; Whatmore *et al.*, 2016)^[64].

Discussion

The observed MRT positivity of 54.0% aligns closely with previous findings in dairy herds by Lindahl *et al.* (2018) (55.6%) and Prasanthi (2022) (70.6%), reflecting substantial shedding of anti-*Brucella* antibodies and active organism excretion into milk (Abnaroodheleh *et al.*, 2023). The lower prevalence observed via RBPT (27.5%) compared to MRT reflects differences in sample substrate dynamics: while RBPT measures systemic circulating IgG1/IgM, MRT detects secretory immunoglobulins that concentrate in mammary secretions during active colonization (Kumar *et al.*, 2016; Kylla *et al.*, 2018). The higher prevalence in Pune (55.8% MRT, 38.83% RBPT) compared to Mumbai (43.3% MRT, 14.92% RBPT) is attributable to larger herd densities, frequent inter-farm animal movements, and variable biosecurity practices in intensive commercial dairy pockets (Abubakar *et al.*, 2010; Mangi *et al.*, 2015). Direct molecular detection via *BCSP31* PCR yielded a 72.39% positivity rate among seropositive cattle, matching rates reported by Garshasbi *et al.* (2014) (73.8%) and Londhe *et al.* (2013) (76.3%). The increased sensitivity of PCR over serology highlights its capacity to bypass anergy, identify early or chronic carrier stages, and detect low-level bacteremia (Kaur *et al.*, 2018; Patel *et al.*, 2018). Milk yielded higher PCR positivity (76.85%) than whole blood (63.63%), supporting previous evidence that *Brucella* targets supramammary lymph nodes and is intermittently shed in high quantities in milk (Al-Mariri and Haj-Mahmoud, 2010; Asgari and Ahmadi, 2021). Amplification of *omp19* (27.6%) and *ure* (33.7%) provides direct evidence of virulent pathogenic strains circulating within these herds (Abo-Ksour, 2018; Fathy *et al.*, 2023)^[34, 50, 1, 31, 32, 3, 36, 22, 35, 26, 47, 6, 12, 2, 21]. The lower recovery of the 2100 bp *ure* and 550 bp *omp19* amplicons compared to the 223 bp *BCSP31* target is consistent with PCR kinetics, where amplifying larger DNA fragments from clinical matrices is more susceptible to target DNA fragmentation, high host-DNA background, and natural PCR inhibitors (calcium ions, fat globules, and proteinases in

milk; heme and anticoagulants in blood) (Al-Shemmari, 2018; Altalhy and Shukri, 2024). The 33.7% *ure* detection rate closely parallels the 33.3% frequency reported by Esmaeel (2019) in milk, confirming the retention of acid-resistance mechanisms necessary for persistent infection. Phylogenetic clustering of Samples 1, 3, and 4 with *B. abortus* S19 alongside the distinct clustering of Sample 2 with virulent wild-type strains reveals epidemiological complexity: S19-like strain recovery from aborted cattle indicates potential vaccine strain persistence, residual virulence, or diagnostic interference in vaccinated herds (Ramadan and Gafer, 2016; Thorat *et al.*, 2017).

Concurrently, the identification of a distinct wild-type clade confirms ongoing transmission of field strains (Pelerito *et al.*, 2020; Tan *et al.*, 2015)^[8, 9, 20, 52, 61, 49, 58]. These findings show that conventional serology alone cannot provide sufficient epidemiological resolution, underscoring the necessity of coupling serological screening with target-specific PCR and sequencing to distinguish field challenge from vaccine-related strains.

Conclusions

- Bovine brucellosis is highly prevalent in dairy herds across the Pune and Mumbai regions of Maharashtra, presenting ongoing risks of production loss and zoonotic spillover.
- MRT serves as an effective, non-invasive method for herd milk screening, while RBPT provides an economical screening tool for serum.
- *BCSP31* PCR offers superior sensitivity for direct pathogen detection in raw milk and blood compared to serological screening alone.
- Amplification of *omp19* and *ure* virulence genes confirms the active circulation of pathogenic *B. abortus* strains equipped for host immune evasion and survival.
- Genomic and phylogenetic analyses reveal the simultaneous presence of both wild-type field strains and S19 vaccine-related strains within the bovine population.
- Implementing combined serological, molecular, and sequence-based surveillance is recommended to improve diagnostic accuracy, support vaccine tracking, and advance regional brucellosis control programs.

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