

STANDARDIZATION OF MULTIPLEX PCR ASSAYS FOR IDENTIFICATION AND VIRULENCE GENE PROFILING OF *BRUCELLA ABORTUS*

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ABSTRACT:

Bovine brucellosis, caused principally by *Brucella abortus*, is endemic in India and causes substantial economic loss. Characterization of *Brucella* is complicated by the genetic homogeneity of the genus and by the need to distinguish field strains from vaccine strains in routine use. *Brucella* lacks classical virulence determinants and depends instead on factors mediating intracellular survival. This study standardized a sequential polymerase chain reaction workflow for genus confirmation, species assignment, vaccine strain discrimination, and virulence gene profiling of *B. Abortus*. Reference strain S99 was characterized phenotypically and biochemically, and genomic DNA was subjected to genus-specific *bcs*31 amplification, *B. Abortus*-specific IS711 amplification, Bruce-ladder multiplex amplification using eight primer pairs, and a four-gene multiplex targeting cyclic beta-1,2-glucan, urease, the type IV secretion system, and outer membrane protein 31. The 16S ribosomal RNA gene was sequenced and compared against reference sequence databases. The strain was catalase, oxidase, and urease positive, reduced nitrate, and showed colony and staining characteristics typical of *Brucella*. The *bcs*31 and IS711 assays amplified products of 223 and 498 base pairs, respectively, confirming the strain as *B. Abortus*. The 16S ribosomal RNA sequence returned identical similarity scores against nine *Brucella* species, confirming genus but not species identity. Bruce-ladder amplification produced the five-fragment profile characteristic of *B. Abortus* field strains, excluding the vaccine strains S19, RB51, and Rev1. The virulence multiplex amplified all four targets, with the 257 base pair *omp*31 product consistently weaker than the others. The workflow permits genus confirmation, species assignment, vaccine strain discrimination, and virulence gene profiling of *B. Abortus* from a single DNA preparation. Primer balance requires further optimization, and analytical sensitivity and specificity remain to be determined before diagnostic application to field isolates.

KEYWORDS: *Brucella abortus*; Bruce-Ladder; Multiplex Polymerase Chain Reaction; Reference Strain; Virulence Genes

INTRODUCTION

Brucellosis is among the most common bacterial zoonoses worldwide, caused by members of the genus *Brucella* — small, non-motile, non-encapsulated, facultatively intracellular Gram-negative coccobacilli of the family Brucellaceae within the order Rhizobiales (Moreno, 2021). Unlike most other Rhizobiales, which are obligate intracellular endosymbionts, *Brucella* is a facultative intracellular pathogen, a distinction that underlies its capacity to cause disease in both animals and humans.

The disease is endemic in the Mediterranean basin, the Arabian Peninsula, parts of Central and South America, and the Indian subcontinent (Pappas et al., 2006). Of the recognised *Brucella* species, *B. melitensis*, *B. suis*, and *B. Abortus* are of greatest consequence for animal and human health (Whatmore et al., 2016), with approximately 500,000 new human infections reported annually (de Figueiredo et al., 2015). Bovine brucellosis is caused predominantly by *B. Abortus*, and widespread prevalence has been documented across several Indian states (Chandramohan et al., 1992; Khurana et al., 2012; Shome et al., 2019). Reported occurrence in India ranges from 10% in marginal herds to 50% in organized farms, with an estimated median economic loss of US \$3.4 billion (Singh et al., 2015), arising principally from abortion, calf loss, reduced milk yield, and infertility.

Accurate identification of *Brucella* to species level is central to both diagnosis and control, but is complicated by the genus's exceptional genetic homogeneity. The 16S rRNA gene, the standard target for bacterial identification, is effectively invariant across *Brucella* species and therefore cannot discriminate between them. Species-level assignment instead requires targets such as the IS711 insertion sequence or the multiplex Bruce-ladder assay, the latter also distinguishing field strains from the vaccine strains *B. Abortus* S19 and RB51 and *B. melitensis* Rev1 (García-Yoldi et al., 2006).

Brucella possesses none of the classical virulence determinants of other bacterial pathogens — no exotoxins,

antiphagocytic capsule, cytolytic enzymes, fimbriae, plasmids, or lysogenic phages — and its lipopolysaccharide poorly stimulates host inflammatory responses (Finlay and Falkow, 1997; Moreno and Moriyón, 2002; Seleem et al., 2008). Virulence depends instead on a specialised set of determinants acting at successive stages of intracellular survival. Cyclic β -1,2-glucan (*c β g*) interacts with host membrane lipid rafts and prevents phagosome–lysosome fusion (Arellano-Reynoso et al., 2005; Yu and Nielsen, 2010). The *virB* operon encodes the type IV secretion system, which translocates effectors required for maturation of the *Brucella*-containing vacuole (Comerci et al., 2001; Cascales and Christie, 2003). Urease (*ure*) confers resistance to the acidic conditions encountered during gastric transit and within the vacuole (Bandara et al., 2007; Sangari et al., 2007). *Omp31* functions as a haemin-binding protein contributing to iron acquisition (Delpino et al., 2006).

Because these determinants act at distinct stages of infection, simultaneous detection is more informative than single-gene approaches, and multiplex PCR panels have been applied to *Brucella* virulotyping in clinical and animal isolates (Derakhshandeh et al., 2013; Razzaq et al., 2014; Naseri et al., 2016; Mirnejad et al., 2017; Rahdar et al., 2018; Awadh et al., 2022). Comparable data from Indian *B. Abortus* isolates remain limited, and no combined workflow linking genus confirmation, species assignment, vaccine strain discrimination, and virulence gene profiling has been described for use in Indian diagnostic laboratories.

The present study was therefore undertaken to standardize a sequential PCR workflow for *Brucella abortus* — comprising genus-specific *bcs31* PCR, species-specific IS711 PCR, Bruce-ladder multiplex PCR, and a four-gene virulence multiplex PCR targeting *c β g*, *ure*, *virB*, and *omp31* — using the reference strain S99.

MATERIALS AND METHODS

Ethical Approval

Blood samples from cattle were collected under a protocol approved by the Institutional Animal Ethics Committee of Nagpur Veterinary College, Nagpur, India (CPCSEA registration 244/GO/ReBi-S/Re-L/2000/CPCSEA). All other work was conducted using a bacterial reference culture.

Study Period and Location

The work was carried out in the Department of Veterinary Pathology, Nagpur Veterinary College, Nagpur, Maharashtra, India, from October 2022 to March 2023.

Bacterial strain and culture conditions

Brucella abortus reference strain S99 (VTCC BAA465) was procured from the National Centre for Veterinary Type Cultures (NCVTC), Hisar, Haryana, India. The strain was maintained on BBL™ *Brucella* Agar and grown in BBL™ *Brucella* Broth (Becton, Dickinson and Company, USA) at 37 °C under 5% CO₂.

Phenotypic and Biochemical Characterization

Colony morphology was recorded on *Brucella* agar. Purity and identity were assessed by Gram's stain and modified Ziehl–Neelsen staining. The strain was subcultured on blood agar and MacConkey agar (HiMedia, India) to confirm absence of haemolysis and of lactose fermentation. Catalase, oxidase, urease, and nitrate reduction tests were performed according to standard procedures (OIE, 2009).

DNA Extraction

Genomic DNA was extracted from broth culture using the HiPurA® Multi-Sample DNA Purification Kit (HiMedia, India) following the manufacturer's protocol for Gram-negative bacteria. Prior to extraction, 1.5 mL of broth culture was heat-inactivated at 80 °C for 1 h. DNA was eluted in 100 μ L of elution buffer, quantified spectrophotometrically at 260 and 280 nm (NanoDrop, Eppendorf, Germany), and stored at –20 °C until use.

Primers

Table 1: All primer sequences were previously published

Assay	Target	Primer sequences (5'→3')	Amplicon (bp)	Reference
Genus PCR	<i>bcs31</i>	CGCGCTTGCCTTTTCAGGTCTG TGGCTCGGTTGCCAATATCAA	/ 223	Baily et al. (1992)
Species PCR	IS711	GACGAACGGAATTTTTCCAATCCC TGCCGATCACTTAAGGGCCTTCAT	/ 498	Bricker and Halling (1994)
Bruce-ladder	<i>wboA</i>	ATCCTATTGCCCCGATAAGG GCTTCGCATTTTCACTGTAGC	/ 1682	García-Yoldi et al. (2006)
Bruce-ladder	<i>omp31</i>	TTTACACAGGCAATCCAGCA GCGTCCAGTTGTTGTTGATG	/ 1071	García-Yoldi et al. (2006)
Bruce-ladder	<i>pda</i>	ACGCAGACGACCTTCGGTAT TTTATCCATCGCCCTGTCAC	/ 794	García-Yoldi et al. (2006)
Bruce-ladder	<i>eryC</i>	GCCGCTATTATGTGGACTGG AATGACTTCACGGTCGTTTCG	/ 587	García-Yoldi et al. (2006)
Bruce-ladder	<i>bp26</i>	GCGCATTCTTCGGTTATGAA CGCAGGCGAAAACAGCTATAA	/ 450	García-Yoldi et al. (2006)
Bruce-ladder	<i>abc</i>	GGAACACTACGCCACCTTGT GATGGAGCAAACGCTGAAG	/ 272	García-Yoldi et al. (2006)
Bruce-ladder	<i>rpsL</i>	CAGGCAAACCTCAGAAGC GATGTGGTAACGCACACCAA	/ 218	García-Yoldi et al. (2006)

Bruce-ladder	<i>crp</i>	CGCAGACAGTGACCATCAAA GTATTCAGCCCCCGTTACCT	/	152	García-Yoldi et al. (2006)
Virulence multiplex	<i>ure</i>	GCTTGCCCTTGAATTCCTTTGTGG ATCTGCGAATTTGCCGGACTCTAT	/	2100	Derakhshandeh et al. (2013)
Virulence multiplex	<i>virB</i>	CGCTGATCTATAATTAAGGCTA TGCGACTGCCTCCTATCGTC	/	881	Naseri et al. (2016)
Virulence multiplex	<i>cβg</i>	GAATTCGCCAATGAGGAAAA ACGATATCGGATGCGAAAAG	/	575	Razzaq et al. (2014)
Virulence multiplex	<i>omp31</i>	GCTGCTCCTGTTGACACCTT GCTGAAATCGAACCCGTAAC	/	257	Razzaq et al. (2014)

Genus-specific *bcs*31 PCR

A 223 bp fragment of the *bcs*31 gene was amplified in a 25 µL reaction containing 12.5 µL of 2× GoTaq® Green Master Mix (Promega, USA), 1.0 µL each of forward and reverse primer (10 pmol/µL), 8.0 µL of nuclease-free water, and 2.5 µL of template DNA. Cycling comprised initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min, with a final extension at 72 °C for 10 min.

To confirm assay performance on clinical material, the *bcs*31 PCR was additionally applied to genomic DNA extracted from whole blood of cattle serologically positive for brucellosis, collected during a parallel seroprevalence survey. DNA was extracted using the HiPurA® Multi-Sample DNA Purification Kit (HiMedia, India) following the manufacturer's protocol for whole blood.

Species-specific IS711 PCR

A 498 bp fragment of the IS711 element specific to *B. Abortus* was amplified using the same reaction composition as above. Cycling comprised initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min 15 s, 55.5 °C for 2 min, and 72 °C for 2 min, with a final extension at 72 °C for 5 min.

Bruce-ladder multiplex PCR

Bruce-ladder PCR was performed using eight primer pairs (García-Yoldi et al., 2006), with minor modification to the cycling conditions. Reactions contained 12.5 µL of 2× GoTaq® Green Master Mix, 4.0 µL of forward primer mix, 4.0 µL of reverse primer mix (each primer at 10 pmol/µL), 2.0 µL of nuclease-free water, and 2.5 µL of template DNA in a total volume of 25 µL. Cycling comprised initial denaturation at 95 °C for 7 min, 30 cycles of 95 °C for 35 s, 64 °C for 45 s, and 72 °C for 3 min, and a final extension at 72 °C for 6 min.

Virulence gene multiplex PCR

Four virulence-associated genes were amplified in a single reaction of identical composition to the Bruce-ladder reaction. Cycling comprised initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

All reactions were performed in a thermal cycler (vapo. protect, Eppendorf, Germany). *B. Abortus* S99 genomic DNA and nuclease-free water served as positive and negative controls, respectively, in every run.

16S rRNA gene sequencing

The 16S rRNA gene amplicon was sequenced bidirectionally on an ABI capillary sequencer. Trace files (.ab1) were imported into CLC Genomics Workbench v21.0.2 (Qiagen, Denmark), trimmed against the UniVec database with a quality score cut-off of 20 and no ambiguous residues permitted, and assembled, with conflicts resolved manually from the chromatograms. The consensus sequence was compared against the NCBI reference RNA sequence database restricted to the genus *Brucella* (taxid: 234) using BLAST.

Agarose Gel Electrophoresis

Amplified products (10 µL) were mixed with 1 µL of 6× loading dye (Promega, USA) and resolved on a 1.5% agarose gel in 0.5× Tris-borate-EDTA buffer containing ethidium bromide (0.5 µg/mL; HiMedia, India). A 100 bp DNA ladder was used for all assays, with an additional 1 kb ladder for assays generating products above 1000 bp. Electrophoresis was carried out at 85 V and 60 mA for 45 min in a horizontal system (Genaxy, India). Gels were visualized on an ultraviolet transilluminator and documented using a gel documentation system (Gel-Pro Analyzer, Syngene, USA).

RESULTS

Phenotypic and Biochemical Characterisation

Strain S99 produced honey-coloured, round, smooth, translucent, glistening colonies on *Brucella* agar. Growth on blood agar was non-haemolytic and colonies on MacConkey agar were non-lactose fermenting. Gram's stain showed Gram-negative coccobacilli, and modified Ziehl–Neelsen staining showed acid-fast organisms. The strain was positive for catalase, oxidase, and urease, and reduced nitrate. All results were consistent with the characteristics described for *Brucella* spp. (OIE, 2009) (**Figure 1**).

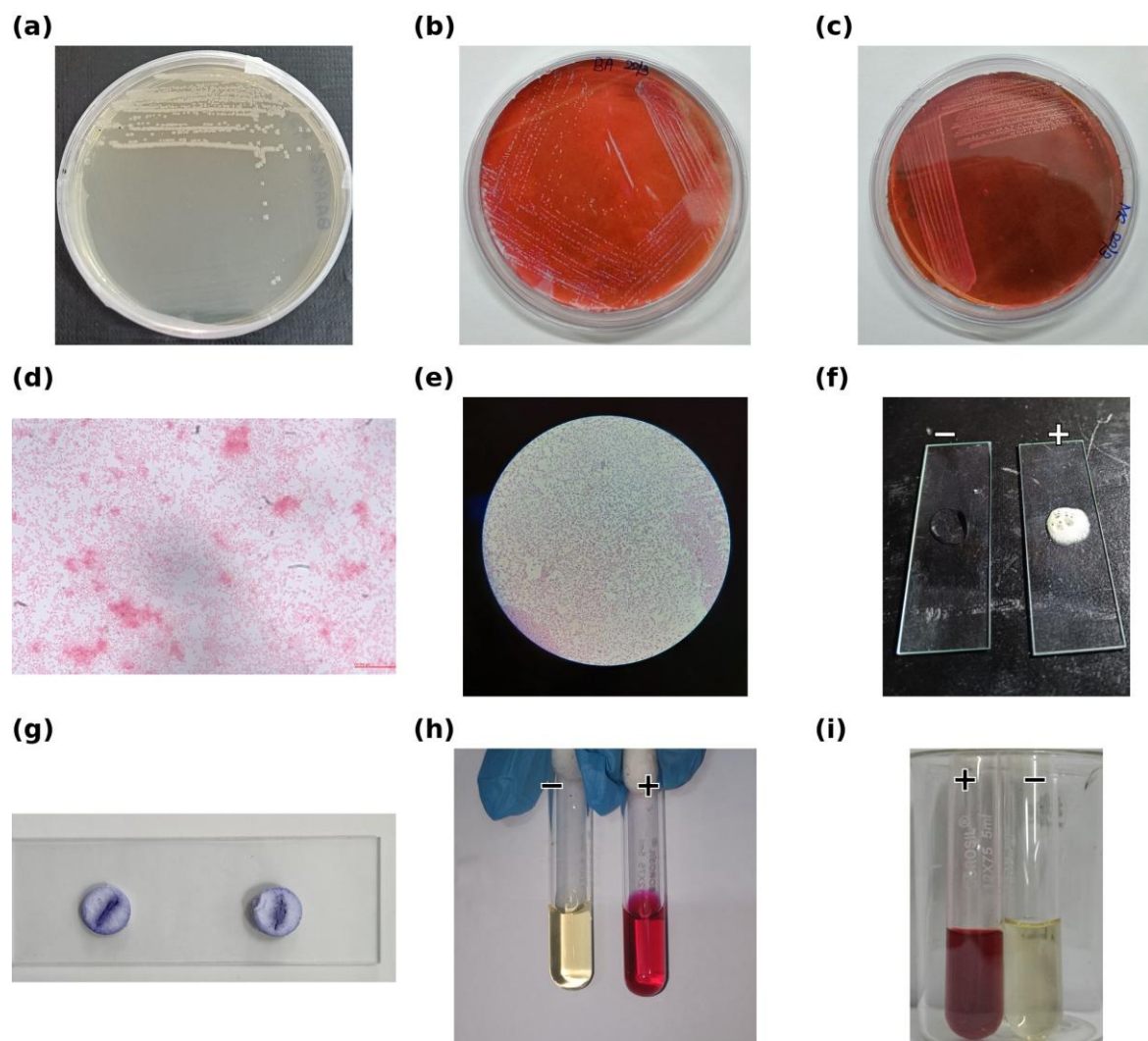


Figure 1. Phenotypic and biochemical characterisation of *Brucella abortus* reference strain S99. (a) Round, convex, translucent, honey-coloured, glistening colonies on *Brucella* agar. (b) Non-haemolytic growth on blood agar. (c) Non-lactose-fermenting colonies on MacConkey agar. (d) Gram stain showing Gram-negative coccobacilli. (e) Modified Ziehl–Neelsen stain showing acid-fast organisms. (f) Catalase test. (g) Oxidase test. (h) Urease test. (i) Nitrate reduction test. In panels f, h and i, + denotes the positive reaction and – the negative control.

Genus and Species Confirmation

The genus-specific *bcs*31 PCR amplified the expected 223 bp product, confirming the isolate as a member of the genus *Brucella* (Figure 2a). The *B. Abortus*-specific IS711 PCR amplified a 498 bp product, confirming species identity (Figure 2b). No amplification was observed in the no-template controls.

The *bcs*31 assay also amplified the expected 223 bp product from blood-derived DNA of five serologically positive cattle, confirming that the assay performs on clinical material as well as on pure culture (Figure 2a).

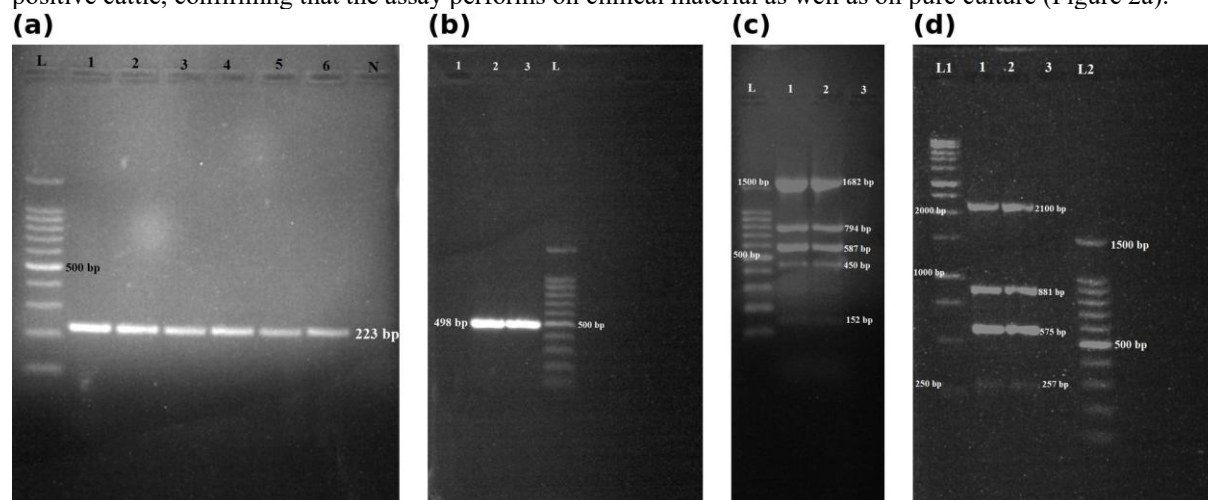


Figure 2. Agarose gel electrophoresis of PCR products from *Brucella abortus* reference strain S99. (a) Genus-

specific *bcs31* PCR (223 bp). Lane L: 100 bp DNA ladder; Lane 1: S99, positive control; Lanes 2–6: DNA extracted from blood of serologically positive cattle; Lane N: negative control. (b) *B. Abortus*-specific IS711 PCR (498 bp). Lane 1: negative control; Lanes 2 and 3: S99; Lane L: 100 bp DNA ladder. (c) Bruce-ladder multiplex PCR. Lane L: 100 bp DNA ladder; Lanes 1 and 2: S99, showing fragments of 1682, 794, 587, 450, and 152 bp; Lane 3: negative control. (d) Virulence gene multiplex PCR. Lane L1: 1 kb DNA ladder; Lanes 1 and 2: S99, showing *ure* (2100 bp), *virB* (881 bp), *cβg* (575 bp), and *omp31* (257 bp); Lane 3: negative control; Lane L2: 100 bp DNA ladder.

16S rRNA Gene Sequencing

The assembled 16S rRNA consensus sequence (1305 bp) returned BLAST hits of identical score against multiple *Brucella* species, including *B. Abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. microti*, *B. ceti*, *B. pinnipedialis*, and *B. neotomae*. The sequence therefore confirmed identity at genus level but did not discriminate between species within the genus.

Bruce-Ladder Multiplex PCR

Bruce-ladder PCR produced the five-fragment profile characteristic of *B. Abortus* field strains, with amplicons of 1682 bp (*wboA*), 794 bp (*pda*), 587 bp (*eryC*), 450 bp (*bp26*), and 152 bp (*crp*) (Figure 2c). The 2524 bp fragment diagnostic of *B. Abortus* RB51 was absent, the 587 bp *eryC* fragment absent in *B. Abortus* S19 was present, and the 218 bp *rpsL* fragment characteristic of *B. melitensis* Rev1 was not amplified. The profile therefore identified S99 as a *B. Abortus* field strain and excluded all three vaccine strains. The 272 bp and 218 bp fragments were not resolved under the electrophoresis conditions used. No amplification was observed in the negative control.

Virulence Gene Multiplex PCR

The virulence gene multiplex amplified all four target genes from S99, producing products of 2100 bp (*ure*), 881 bp (*virB*), 575 bp (*cβg*), and 257 bp (*omp31*) (Figure 2d). The three larger products were clearly resolved and of strong intensity. The 257 bp *omp31* product was consistently amplified but gave a band of markedly lower intensity than the other three targets. Use of a 1 kb ladder alongside the 100 bp ladder permitted accurate sizing across the 257–2100 bp range spanned by the assay. No amplification was observed in the no-template control.

DISCUSSION

The workflow standardized here moves from genus to species to strain type to virulence profile, each step resolving what the preceding one cannot. The *bcs31* gene is conserved across the genus and is widely used for genus-level detection of *Brucella* from cultures and clinical material (Baily et al., 1992; Bricker, 2002). The IS711 element, present in multiple copies with species-specific flanking regions, permits species assignment (Bricker and Halling, 1994), and the Bruce-ladder assay adds discrimination of field strains from the vaccine strains in routine use (García-Yoldi et al., 2006).

The 16S rRNA results illustrate why this stepwise approach is necessary. The consensus sequence returned identical BLAST scores against nine *Brucella* species, which is expected given the near-complete conservation of the 16S rRNA gene across the genus. 16S sequencing is therefore adequate for genus confirmation but cannot be used for species assignment in *Brucella*, and reports relying on it alone for speciation should be interpreted with caution.

The Bruce-ladder profile obtained was diagnostic. *Brucella abortus* field strains yield fragments of 1682, 794, 587, 450, and 152 bp. *B. Abortus* RB51 is distinguished by a 2524 bp fragment arising from an IS711 insertion within *wboA*, together with loss of the 1682 bp fragment; *B. Abortus* S19 lacks the 587 bp *eryC* fragment owing to a deletion in the erythritol catabolism region; and *B. melitensis* Rev1 is identified by amplification of a 218 bp *rpsL* fragment (García-Yoldi et al., 2006). The profile obtained from S99 matched the field strain pattern and excluded all three vaccine strains in a single reaction. This capacity is of practical value in India, where S19 vaccination is used and where serological positivity cannot distinguish vaccinated from infected animals.

The four genes selected for the virulence multiplex represent functionally distinct and complementary stages of intracellular survival: membrane interaction and inhibition of phagosome–lysosome fusion (*cβg*), acid resistance during gastric transit and within the *Brucella*-containing vacuole (*ure*), effector translocation through the type IV secretion system (*virB*), and iron acquisition (*omp31*). Assays targeting several determinants simultaneously are therefore more informative than single-gene approaches.

Multiplex virulotyping of *Brucella* has been reported from several settings. Naseri et al. (2016) screened eleven virulence-associated genes across 57 *B. melitensis* isolates from human blood cultures, detecting ten in all isolates. Mirnejad et al. (2017) targeted six genes across 78 isolates of *B. melitensis* and *B. Abortus*, reporting frequencies from 74.4% for *ure* to 100% for *manA*. Rahdar et al. (2018) applied a five-gene panel to synovial fluid samples, detecting *cβg*, *virB*, and *omp31* in all *B. Abortus* and *B. melitensis* samples. Razzaq et al. (2014) and Awadh et al. (2022) reported comparable panels from human clinical isolates in Iraq, with lower frequencies for some targets. These studies consistently show high but variable gene content, indicating that virulence determinants differ between species, strains, and geographical origin. The assay standardized here condenses four of these determinants into a single reaction, reducing reagent consumption and turnaround time relative to sequential single-gene PCR.

The low intensity of the 257 bp *omp31* product merits comment. In a multiplex containing a 2100 bp target, the smallest amplicon competes for polymerase and nucleotides against products requiring substantially longer synthesis, and preferential amplification of one target at the expense of another is a recognised limitation of

multiplex design. Adjustment of the relative primer concentrations would be expected to improve the balance, and this optimization should be completed before the assay is applied to field material, where template concentrations will be lower than from pure culture.

LIMITATIONS

The assays were standardized using a single reference strain, and performance on field isolates of diverse geographical and host origin remains to be established. Analytical sensitivity, expressed as a limit of detection, and analytical specificity against non-*Brucella* organisms were not determined, and both are required before the workflow can be recommended for diagnostic use. Detection of a gene by PCR indicates its presence but not its expression or functional contribution to virulence; transcriptional analysis or infection models would be needed to establish that. Finally, no field isolates were recovered during the associated sampling, so the virulence gene content of circulating Indian *B. Abortus* strains remains undetermined.

CONCLUSION

A sequential PCR workflow was standardized for the identification and virulence gene profiling of *Brucella abortus*, comprising genus-specific *bcs31* PCR, species-specific IS711 PCR, Bruce-ladder multiplex PCR for strain typing and vaccine strain discrimination, and a four-gene multiplex PCR targeting *cβg*, *ure*, *virB*, and *omp31*. All assays performed as expected against the reference strain S99, which was confirmed as a *B. Abortus* field strain carrying all four virulence determinants. The *omp31* amplicon requires further optimization of primer balance. Subject to determination of analytical sensitivity and specificity, the workflow offers a rapid and cost-effective approach for characterizing *B. Abortus* isolates from brucellosis-endemic regions of India.

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