Visual Detection of *Brucella* spp. in Spiked Bovine Semen Using Loop-Mediated Isothermal Amplification (LAMP) Assay

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Abstract Several pathogens including *Brucella* spp. are shed in semen of infected bulls and can be transmitted to cows through contaminated semen during artificial insemination. The present study reports *omp2a* and *bcs31* gene based loop-mediated isothermal amplification (LAMP) assays for detection of *Brucella* genomic DNA in semen from infected bulls. The positive results could be interpreted visually by change in colour of reaction mixture containing hydroxyl naphthol blue (HNB) dye from violet to sky blue. LAMP assays based on *omp2a* and *bcs31* could detect as little as 10 and 100 fg of *B. abortus* S19 genomic DNA, respectively. Sensitivity of *omp2a* and *bcs31* LAMP assays for direct detection of organisms in bovine semen was 2.28×10^1 CFU and 2.28×10^2 CFU of *B. abortus* S19 in spiked bovine semen, respectively. The *omp2a* LAMP assay was found equally sensitive to TaqMan probe based real-time PCR and 100 times more sensitive than conventional PCR in identifying *Brucella* in spiked

semen. The diagnostic applicability of the *omp2a* LAMP assay was evaluated with seventy-nine bovine semen samples and results were re-evaluated through TaqMan probe based real-time PCR and conventional PCR. Taken together, the *omp2a* LAMP assay is easy to perform, rapid and sensitive in diagnosis of *Brucella* spp. in bovine semen.

Keywords Loop-mediated isothermal amplification assay · Bovine brucellosis · Bovine semen · *omp2a* · *bcs31*

Introduction

Brucellosis is an infectious and zoonotic disease of animals and humans caused by bacteria belonging to the genus *Brucella*. In cattle, brucellosis is usually caused by *Brucella abortus* (*B. abortus*), less frequently by *B. melitensis* and occasionally by *B. suis* [1]. The disease is characterised by abortion, retention of placenta, orchitis and epididymitis [1]. The *Brucella* pathogens are excreted through semen of infected bulls and the disease may be transmitted to healthy cows through *Brucella* contaminated semen used in artificial insemination (AI) [2]. Thus, transmission of *Brucella* pathogen, through frozen semen may be a threat to national cattle herd, where AI is used as a major breeding tool for genetic improvement. This is also a matter of concern for countries involved in international trading of frozen semen for overseas distribution of superior livestock germplasm. Diagnostic methods like microbiological isolation and identification of organisms and nucleic acid amplification based techniques have been used for detection of *Brucella* pathogens in bull semen [1]. Although microbiological isolation is the gold standard for *Brucella* detection in semen, this method is time consuming and there is risk to laboratory personnel. A sensitive nucleic acid based

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method, polymerase chain reaction (PCR) has been used in detection of *Brucella* genomic DNA in bovine semen [2–4]. Minimal requirements like expensive PCR machines and skilled personnel (for reaction set up and result analysis) limit PCR technique being used as a routine diagnostic test for Brucellosis under field condition and less equipped laboratories at breeding bull stations.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid sequence amplification based assay, amplifies target DNA under isothermal condition in the range of 60–65 °C with high specificity, sensitivity and rapidity [5]. LAMP amplified products can be detected either visually with naked eyes by observing post amplification turbidity of reaction mixture or by using nucleic acid intercalating dyes [6]. Because of high target specificity, sensitivity, simplicity and rapidity, LAMP assay has been widely used in laboratories as a nucleic acid sequence based test to detect microorganisms causing infectious diseases in humans, animals and plants, identification of genetically modified products and tumours and embryo sexing [7].

LAMP assays have been reported for detection of *Brucella* organisms in milk, blood and organs of aborted foetuses [8–13]. In this study, we report LAMP assays targeting *omp2a* and *bcp31* genes for detection of *Brucella* in bull semen. The *omp2a* (Outer membrane protein 2a) gene encodes porin, a pore forming protein and responsible for efficient diffusion of sugar into cells [14]. The *bcp31* (*Brucella* cell surface protein 31) gene encodes a 31 kDa immunogenic protein. The *bcp31* is conserved among many *Brucella* spp [15]. Diagnosis sensitivity of standardised LAMP assays (*omp2a* and *bcp31*) were compared with that of conventional PCR and TaqMan probe based real-time PCR. This is the first report of visual detection of *Brucella* spp. in the bovine semen by *omp2a* LAMP.

Materials and Methods

Bacterial Strains and Semen Samples

Four *Brucella* species and thirteen non-*Brucella* species of bacteria are used in this study (Table S1). Trypticase soy agar (Difco, USA) was used to grow *B. abortus* S19.

A total of seventy-nine semen samples were collected from breeding bulls housed in semen collection centres and organised farms. All bulls were found sero-negative to anti-*Brucella* antibodies in rose bengal plate test (RBPT).

Primer Design

The coding sequences for *Brucella* spp. specific genes, namely, *omp2a* (GenBank accession no. M26034) and *bcp31* (GenBank accession no. M20404) were aligned

with available sequences in GenBank. The nucleotide sequence region of genes found conserved among *Brucella* genus members were selected as targets for designing LAMP primers. Online software program, “Primer Explorer V4” (<http://primerexplorer.jp/e>) was used for designing primers. For each target, six primers, including outer primers (F3 and B3), loop primers (LF and LB) and inner primers (FIP and BIP) were designed (Fig S1a and S1b, Table S2). All primers were purchased from Integrated DNA Technologies (IDT, USA).

Bacterial Genomic DNA Isolation

Reaction conditions of LAMP assays were optimised by using genomic DNA (as template) isolated from inactivated *B. abortus* S19 using CTAB with slight modifications [16]. The cell pellet from 1.5 mL culture was re-suspended in 567 µL of TE buffer (pH 8.0). The cells were incubated for 1 h at 37 °C with 30 µL of sodium dodecyl sulphate (10 %) and 3.0 µL of Proteinase K (20 mg/mL). The cell lysate was further incubated at 65 °C for 10 min with 100 µL of 5 M NaCl and 80 µL of CTAB/NaCl solution. Following incubation, extracts were purified using phenol: chloroform: isoamyl alcohol (25:24:1). Then after, genomic DNA was precipitated from aqueous phase by adding 1/10 volume of 7.5 % ammonium acetate and double volume of absolute ethanol, washed twice with 70 % ethanol, air-dried and dissolved in 100 µL of TE buffer.

To get pure form of genomic DNA, QIAamp DNA Mini Kit (Qiagen, Hilden) was used to isolate total genomic DNA from bovine semen samples and *B. abortus* S19 spiked bovine semen as per manufacturer’s protocol. A volume of 10 µL bovine semen was used for genomic DNA isolation and DNA was eluted in 20 µL elution buffer.

Optimisation of LAMP Assays

Both *omp2a* and *bcp31* LAMP assays were optimized by varying concentration of reaction components and reaction conditions. The assays were evaluated at different amplification temperatures (60–68 °C), reaction time (15–60 min), concentration of MgSO₄ (0–6.4 mM) and dNTPs (0 to 2 mM). The LAMP assay reactions were performed in a PCR machine (S-24 Satellite thermal cycler, Quanta Biotech). The optimized reaction conditions for *omp2a* constituted of the following components: 0.2 µM each of outer primers (F3 and B3), 1.6 µM each of inner primers (FIP and BIP), 0.8 µM each of loop primers (LF and LB), 1.2 M betain (Sigma), 3.2 mM MgSO₄ (Amresco), 1.2 mM dNTPs mix (Fermentas), 1X ThermoPol reaction buffer (20 mM Tris–HCl, 10 mM KCl, 2 mM MgSO₄, 10 mM (NH₄)₂SO₄ and 0.1 % Triton

X-100) (NEB), 120 μ M hydroxynaphthol blue (HNB) dye (Sigma) and target DNA (50–100 ng) in a 25 μ L reaction volume. The reaction mixture was heated at 95 °C for 5 min for genomic DNA (template) denaturation and immediately cooled on ice for 2 min. The reaction tubes were briefly centrifuged and 8 units large fragment of *Bst* DNA polymerase (NEB) was added to the reaction mixture and incubated at 63 °C for 60 min for amplification reaction to happen. Finally the reaction was terminated by heating at 80 °C for 5 min. The optimised condition for *bcs31* LAMP assay was similar to *omp2a* assay except for 2.4 mM MgSO_4 and 0.8 mM dNTPs.

Detection of Amplified LAMP Products

The amplified products of LAMP reactions were analysed visually by naked eye as well as by agarose gel electrophoresis. With HNB dye, the colour of the reaction mixture changes from violet to sky blue in case of positive samples and remains unchanged (i.e. violet) for negative samples. The results were further confirmed by resolving 3 μ L of LAMP product on 2 % agarose gel.

Sensitivity and Specificity of LAMP Assays

The sensitivity of *omp2a* and *bcs31* LAMP assays was performed with and without HNB dye in the reaction mixture. The detection limits of *omp2a* and *bcs31* LAMP assays were determined by limiting dilution method. The purified *B. abortus* S19 genomic DNA was serially diluted (tenfold) with nuclease free water and each dilution was put to LAMP reaction to determine detection limit. Further to determine the sensitivity of LAMP assays, semen from a healthy bull (*Brucella* negative) was spiked with known number of *B. abortus* S19 cells. Diagnosis sensitivity of the LAMP assays were compared with reported conventional PCR and TaqMan probe based real time PCR methods.

The specificity of *omp2a* and *bcs31* LAMP assays was analysed with total genomic DNA isolated from *Brucella* and non-*Brucella* spp. (Table S1). The sensitivity and specificity experiments were repeated thrice.

Conventional PCR and TaqMan Probe Based Real-Time PCR Assays

The conventional PCR was performed by using BMEI0535f and BMEI0536r primers from reported Brucella ladder multiplex PCR assay [1]. PCR reactions were carried out in a thermal cycler (S-24 Satellite, Quanta Biotech), in 50 μ L of reaction volume consisting of primers (each of 10 pico moles), 1X PCR master mix (ThermoFischer Scientific) and genomic DNA template (at various concentrations). The reaction mixture was

subjected to initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation, primer annealing and extension at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min respectively and final extension at 72 °C for 5 min. The PCR products were analysed on 1.5 % agarose gel.

The TaqMan probe based real-time PCR was carried out as reported earlier [17]. In brief, the 25 μ L reaction volume consisted of 0.2 μ M of probe, 0.4 μ M each of primers and 0.5 μ L of isolated DNA in 1X probe Mastermix (Sigma, EU). The reaction mixture was initially denatured at 94 °C for 2 min. followed by amplification reactions included 40 cycles of denaturation at 94 °C for 15 s, annealing and extension at 60 °C for 1 min. The amplification was carried out in a real-time PCR machine (Mx3005P, Agilent).

Screening of Bovine Semen Samples

Seventy-nine bovine semen samples along with positive and negative controls were subjected to *omp2a* LAMP assay, conventional PCR and TaqMan probe based real-time PCR. Genomic DNA isolated from spiked semen of dilutions 2.28×10^1 CFU and 2.28×10^3 CFU were used as positive controls for *omp2a* LAMP assay/TaqMan probe based real time PCR and conventional PCR respectively. Genomic DNA isolated from semen of *Brucella* negative bulls were used as template for negative controls. Each test was performed three times for all semen samples in order to avoid false positive and false negative results.

Results and Discussion

The present study was aimed at developing a diagnostic test for rapid detection of *Brucella* spp. in bovine semen. Two LAMP assays targeting conserved regions from *omp2a* and *bcs31* genes of *Brucella* spp were analysed and optimized. Amplification was observed from 60–68 °C. However, 63 °C was chosen as optimum temperature for LAMP assays (data not shown). The amplified products could be visualized as early as 30 min of incubation at 63 °C, however, higher amplification was observed after 60 min (data not shown). LAMP assay targeting *omp2a* gene produced best result with 1.2 mM dNTPs and 3.2 mM MgSO_4 while *bcs31* LAMP was optimised with 0.8 mM of dNTPs and 2.4 mM of MgSO_4 (data not shown). Positive LAMP reactions were visually identified by change in colour of reaction mixture from violet to sky blue. In LAMP negative reactions, the colour of reaction mixture remained violet (Fig S2a). Amplified products in positive reactions exhibited typical DNA laddering pattern on agarose gel (Fig S2b). A positive LAMP reaction can also be detected by visualising turbidity generated due to

accumulation of reaction by-product magnesium pyrophosphate [18]. But detection of turbidity requires some skill and intense observation to distinguish positive from negative LAMP results. In this study, inclusion of HNB dye in reaction mixture before amplification reaction has facilitated visual distinction between positive and negative LAMP reactions. There is no requirement of post-amplification electrophoretic analysis of samples. Gradual depletion of magnesium ion during amplification reaction is the reason behind change of pre-amplification violet colour of reaction mixture to post-amplification sky blue colour in a positive LAMP reaction [6]. DNA intercalating

dyes like SYBR green I and picogreen can be used for detecting amplified product [19, 20]. But it requires a UV light source for visualisation. Moreover, these dyes are added to reaction mixture on completion of amplification reaction thus increasing the possibility of laboratory contamination [21]. HNB dye based *omp2a* and *bcs31* LAMP assays are more of closed tube approach. There is no need of opening the tubes after reaction to add dye and thereby, minimizes cross contamination.

The specificity of the LAMP assays was analysed by using genomic DNA isolated from *Brucella* and non-*Brucella* species as targets for amplification reactions. LAMP

Fig. 1 Analytical sensitivity of *omp2a* and *bcs31* LAMP assays (a, b) in comparison with TaqMan probe based real-time PCR (c) and conventional PCR (d) for detection of *Brucella abortus* S19 organism. The bovine semen spiked with *B. abortus* S19 (from

2.28×10^6 to 2.28×10^{-1} CFU) was used for total genomic DNA isolation and used for amplification. Lane L GeneRuler 100 bp plus ladder. Lane M λ DNA double digested with *Eco*RI and *Hind*III Marker. Lane NTC No template control

reaction mixtures were turned sky blue for all *Brucella* strains and remained violet for non-*Brucella* species. The results were also confirmed through agarose gel electrophoresis. The non-*Brucella* bacterial pathogens like *Leptospira* spp., *Coxiella burnetii*, *Mycobacterium avium* subsp *paratuberculosis* and *Mycoplasma mycoides* ssp. *Mycoides* can be transmitted through semen [22]. These organisms didn't show cross reactivity in both *omp2a* and *bcs31* LAMP assays (Fig S3a and S3b), confirming their specificity to *Brucella* species.

The sensitivity of *omp2a* and *bcs31* LAMP assays was remained same with and without HNB dye in the reaction mixture (data not shown). This indicated that HNB dye didn't interfere in amplification process. Sensitivity of the LAMP assays was determined by using *Brucella* genomic DNA as template. The *omp2a* and *bcs31* LAMP assays could detect as little as 10 fg and 100 fg of *Brucella* genomic DNA respectively (data not shown). In conventional PCR, the detection limit was found 10 pg of *Brucella* genomic DNA (data not shown). Thus, sensitivity of both LAMP assays was found 100 times higher to that of conventional PCR when pure genomic DNA was used as template.

Sensitivity of the LAMP assays for direct detection of *Brucella* organisms in semen was determined by spiking bovine semen with *B. abortus* S19. The *omp2a* and *bcs31* LAMP assays could detect 2.28×10^1 CFU (Fig. 1a) and 2.28×10^2 CFU (Fig. 1b) of *B. abortus* S19 respectively in spiked semen. The results evaluated *omp2a* LAMP assay as ten times more sensitive than *bcs31* LAMP assay in detecting *Brucella* in spiked semen. The sensitivity of *omp2a* LAMP assay suggests its usefulness in detecting as little as 23 *Brucella* organisms in semen. The sensitivity of *omp2a* and *bcs31* LAMP assays was also compared with conventional PCR and TaqMan probe based real-time PCR using total genomic DNA isolated from each dilutions of *B. abortus* S19 spiked semen. The TaqMan probe based real-time PCR could detect 2.28×10^1 CFU of *B. abortus* S19 (Fig. 1c) while the sensitivity of the conventional PCR was to detect 2.28×10^3 CFU of *B. abortus* S19 in spiked semen (Fig. 1d). These results indicated that the LAMP assays were 10–100 times more sensitive than conventional PCR in detecting *Brucella* in semen. Presence of amplification inhibitory components in bovine semen [2, 3] or presence of large amount of host genomic DNA [25] or lesser number of *Brucella* organisms in higher dilutions of spiked semen (below detection limit) could be reasons for lower sensitivity of conventional PCR. However, sensitivity of the *omp2a* LAMP assay was equivalent to TaqMan probe based real time PCR. The IS711 TaqMan probe based real time PCR has been reported as a highly sensitive method in detecting less than 10 fg of genomic

DNA (three genome copies) of *Brucella* organisms [17, 23, 24].

Seventy-nine semen samples collected from breeding bulls were tested by *omp2a* LAMP assay. All samples were found negative with LAMP assay as evident from violet colour of the reaction mixture after amplification reaction and gel electrophoresis analysis of LAMP products. These samples were also found negative with conventional PCR and TaqMan probe based real-time PCR. *Brucella* organisms could not be isolated on trypticase soy agar (TSA) from all the seventy-nine semen samples. This might be due to regular screening and segregation of infected bulls at germ plasm centres where bulls are used for semen collection. The negative result further proves the high specificity of *omp2a* LAMP assay to *Brucella* spp.

Conclusions

In summary, HNB dye based *omp2a* LAMP assay could be used as sensitive, specific and user-friendly test to detect *Brucella* spp. in bovine semen at organized farms and semen collection centres with limited laboratory facilities. In future more number of field semen samples need to be subjected to *omp2a* LAMP assay in order to validate the test for detection of *Brucella* spp. in bovine semen samples.

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Compliance with Ethical Standards

Conflict of interest Authors declare that they have no conflict of interest.

References

1. OIE (2009) Bovine brucellosis. OIE Terrestrial Manual 2009, Chapter 2.4.3
2. Amin AS, Hamdy MER, Ibrahim AK (2001) Detection of *Brucella melitensis* in semen using the polymerase chain reaction. Vet Microbiol 83:37–44. doi:10.1016/S0378-1135(01)00401-1
3. Vinodh R, Raj GD, Govindarajan R, Thiagarajan V (2008) Detection of *Leptospira* and *Brucella* genomes in bovine semen using polymerase chain reaction. Trop Anim Health 40:323–329. doi:10.1007/s11250-007-9110-5
4. Junqueira Junior DG, Rosinha GMS, Carvalho CEG, Oliveira CE, Sanches CC, Lima-Ribeiro AMC (2013) Detection of *Brucella* spp. DNA in the semen of seronegative bulls by polymerase chain reaction. Transbound Emerg Dis 6:376–377. doi:10.1111/j.1865-1682.2012.01347.x

5. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28:E63. doi:[10.1093/nar/28.12.e63](https://doi.org/10.1093/nar/28.12.e63)
6. Goto M, Honda E, Ogura A, Nomoto A, Hanak KI (2009) Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques* 46:167–172. doi:[10.2144/000113072](https://doi.org/10.2144/000113072)
7. Fu S, Qu G, Guo S, Ma L, Zhang N, Zhang S, Gao S (2011) Applications of loop-mediated isothermal DNA amplification. *Appl Microbiol Biotechnol* 163:845–850. doi:[10.1007/s12010-010-9088-8](https://doi.org/10.1007/s12010-010-9088-8)
8. Karthik K, Rathore R, Thomas P, Arun TR, Viswas KN, Dhama K, Agarwal RK (2014) New closed tube loop mediated isothermal amplification assay for prevention of product cross-contamination. *MethodsX* 1:137–143. doi:[10.1016/j.mex.2014.08.009](https://doi.org/10.1016/j.mex.2014.08.009)
9. Karthik K, Rathore R, Thomas P, Arun TR, Viswas KN, Manjunathachar HV, Agarwal RK, Dhama K (2014) Loop mediated isothermal amplification (LAMP) test for specific and rapid detection of *Brucella abortus* in cattle. *Vet Q* 34:174–179. doi:[10.1080/01652176.2014.966172](https://doi.org/10.1080/01652176.2014.966172)
10. Perez-Sancho M, Garcia-Seco T, Arroqante L, Garcia N, Martinez I, Diez-Guerrier A, Perales A, Goyache J, Dominguez L, Alvarez J (2013) Development and evaluation of an *IS711*-based loop mediated isothermal amplification method (LAMP) for detection of *Brucella* spp. on clinical samples. *Res Vet Sci* 95:489–494. doi:[10.1016/j.rvsc.2013.05.002](https://doi.org/10.1016/j.rvsc.2013.05.002)
11. Song L, Li J, Hou S, Li X, Chen S (2012) Establishment of loop-mediated isothermal amplification (LAMP) for rapid detection of *Brucella* spp. and application to milk and blood samples. *J Microbiol Methods* 90:292–297. doi:[10.1016/j.mimet.2012.05.024](https://doi.org/10.1016/j.mimet.2012.05.024)
12. Lin GZ, Zheng FY, Zhou JZ, Gong XW, Wang GH, Cao XA, Qiu C (2011) Loop-mediated isothermal amplification assay targeting the *omp25* gene for rapid detection of *Brucella* spp. *Mol Cell Probes* 25:126–129. doi:[10.1016/j.mcp.2011.01.001](https://doi.org/10.1016/j.mcp.2011.01.001)
13. Ohtsuki R, Kawamoto K, Kato Y, Shah MM, Ezaki T, Makino SI (2008) Rapid detection of *Brucella* spp. by the loop-mediated isothermal amplification method. *J Appl Microbiol* 104:1815–1823. doi:[10.1111/j.1365-2672.2008.03732.x](https://doi.org/10.1111/j.1365-2672.2008.03732.x)
14. Marquis H, Ficht TA (1992) The *omp2* gene locus of *Brucella abortus* encodes two homologous outer membrane proteins with properties characteristic of bacterial porins. *Infect Immun* 61:3785–3790
15. Halling SM, Detilleux PG, Tatum FM, Judge BA, Mayfield JE (1991) Deletion of the BCSP31 gene of *Brucella abortus* by replacement. *Infect Immun* 59:3863–3868
16. Wilson K (2001) Preparation of genomic DNA from bacteria. *Curr Protoc Mol Biol* 00: 1: 2.4:2.4.1–2.4.5. doi: [10.1002/0471142727.mb0204s56](https://doi.org/10.1002/0471142727.mb0204s56)
17. Hinic V, Brodar I, Thomann A, Cvetnic Z, Makaya PV, Frey J, Abril C (2008) Novel identification and differentiation of *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* suitable for both conventional and real-time PCR systems. *J Microbiol Methods* 75:375–378. doi:[10.1016/j.mimet.2008.07.002](https://doi.org/10.1016/j.mimet.2008.07.002)
18. Mori Y, Nagamine K, Tomita N, Notomi T (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun* 289:150–154. doi:[10.1006/bbrc.2001.5921](https://doi.org/10.1006/bbrc.2001.5921)
19. Dukes JP, King DP, Alexandersen S (2006) Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. *Arch Virol* 151:1093–1106. doi:[10.1007/s00705-005-0708-5](https://doi.org/10.1007/s00705-005-0708-5)
20. Parida M, Horioka K, Ishida H, Dash PK, Saxena P, Jana AM, Islam MA, Inoue S, Hosaka N, Morita K (2005) Rapid detection and differentiation of dengue virus serotypes by a real time reverse transcription loop-mediated isothermal amplification assay. *J Clin Microbiol* 43:2895–2903. doi:[10.1128/JCM.43.6.2895-2903.2005](https://doi.org/10.1128/JCM.43.6.2895-2903.2005)
21. Almasi MA, Ojaghkandi MA, Hemmatabadi A, Hamidi F, Aghaei S (2013) Development of colorimetric loop-mediated isothermal amplification assay for rapid detection of the tomato yellow leaf curl virus. *J Plant Pathol Microb* 4:153. doi:[10.4172/2157-7471.1000153](https://doi.org/10.4172/2157-7471.1000153)
22. Daniel Givens M, Marley MSD (2008) Pathogens that cause infertility of bulls or transmission via semen. *Theriogenology* 70:504–507. doi:[10.1016/j.theriogenology.2008.05.033](https://doi.org/10.1016/j.theriogenology.2008.05.033)
23. Bounaadja L, Albert D, Chenais B, Henault S, Zygmunt MS, Poliak S, Garin-Bastuji B (2009) Real-time PCR for identification of *Brucella* spp.: a comparative study of IS711, bcs31 and per target genes. *Vet Microbiol* 137:156–164. doi:[10.1016/j.vetmic.2008.12.023](https://doi.org/10.1016/j.vetmic.2008.12.023)
24. Newby DT, Hadfield TL, Roberto FF (2003) Real-time PCR detection of *Brucella abortus*: a comparative study of SYBR green I, 59-exonuclease, and hybridization probe assays. *Appl Environ Microbiol* 69:4753–4759. doi:[10.1128/AEM.69.8.4753-4759.2003](https://doi.org/10.1128/AEM.69.8.4753-4759.2003)
25. Cogswell FB, Banta CE, Hughes TG, Gu Y, Philipp MT (1996) Host DNA can interfere with detection of *Borrelia burgdorferi* in skin biopsy specimens by PCR. *J Clin Microbiol* 34:980–982