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**Communication**

[*Comunicação*]

# A real-time quantitative PCR based on molecular beacon for detecting *Brucella* infection

[*PCR quantitativo em tempo real, baseado em guia molecular para detecção de infeção por Brucella]*

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Brucellosis is a zoonotic infection caused by *Brucella* resulting in reproductive failure in animals and febrile diseases in humans (Boschiroli *et al.*, 2001), and is endemic in areas such as the Middle East, Western Asia, Africa, and South America (Pappas *et al.*, 2006). The prevalence of human brucellosis in China, especially in Xinjiang and Inner Mongolia, has significantly increased in the past decades (Piao *et al.*, 2018).

Based on sole phenotypic characterization using a range of bacteriological and biochemical tests, *Brucella* was generally classified into six species including *Brucella melitensis, Brucella abortus, Brucella suis, Brucella canis, Brucella ovis* and *Brucella neotomae (*Christopher *et al., 2010)*. Over recent years, the number of species has increased to 10 as several new *Brucella* strains had been isolated from marine mammals, rodents, and infected human breast implant (Foster *et al.*, 2007, Scholz *et al.*, 2008, 2010).

The brucellosis in humans has a diverse range of clinical symptoms, and the most important of them was undulant fever and arthrodynia (Franco *et al.*, 2007, Pappas *et al.*, 2005). A substantial proportion of patients were accompanied with splenomegaly and/or hepatomegaly. When the disease becomes chronic, it would affect almost all organs and result in a complicated syndrome including spondylitis, endocarditis, and meningoencephalitis (Pappas *et al*., 2005).

Generally, the diagnostic methods of brucellosis included direct microbiological analysis and indirect tests such as serological tests. Bacterial culture is a gold criterion for a decisive diagnosis of disease, but it requires a long time, and has low sensitivity (Christopher *et al*., 2010). The serological test methods such as the standard agglutination test (SAT) and the Rose Plate Bengal Test (RBPT) were used to detected *Brucella* antibodies, but the findings of serological methods have possibly been confused by vaccines, repeated exposure to antigenic stimuli, or cross-reaction with antigens other than those from *Brucella (*Franc *et al., 2018)*.

Detection of Brucella by molecular assay is highly sensitive compared to bacterial cultures and more specific in comparison with the serological experiments. PCR method is increasingly used as a high-confidence, cost-effective, sensitive, specific, and rapid method to identify the specific sequences of the *Brucella* genus, species, and biotypes and is also used as a surveillance tool to analyze outbreaks of brucellosis (Saini *et al.*, 2017).

Molecular beacons (MBs) are labeled single-stranded oligonucleotide which possess a stem-and-loop structure (Sokol *et al.*, 1998). The loop is single strain complemented to its target sequence, while the stem is double strain formed by two complementary arms of 5 to 7bp to stabilize the loop. The end of one arm is labeled with fluorophore, and the end of the other arm is attached with a quencher. The binding of the loop

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to its target sequence opens the stem and removes the quencher from the fluorophore resulting in fluorescence (Stryer, 1978). MB generates signals only in the presence of the target. In order to overcome the problems causing misleadings and suspicion, a new method for diagnosis of *Brucella* infections is required. In this study, a real-time quantitative PCR method based on molecular beacon was established and evaluated. Diagnostic value was also investigated by comparing with bacteria culture and serological tests.

The nucleotide sequences of the gene coding for the outer membrane protein OMP-2 reported for *B. abortus , B. melitensis, B. ovis, B. suis, and B. canis* were obtained from the GenBank database located at the National Center for Biotechnology Information of the National Library of Medicine (Bethesda, Md.), and all the alignments were carried out using Mfold (Version3.0). Primers and MB probe on the most conserved regions were designed using Primer 5 and Beacon 7 softwares, and synthesized by GenScript BioTech Company (Nanjing, China). The forward primer (JPF) sequence is 5'-atcaagagccttctccttgg-3', the reverse primer (JPR) sequence is 5'-gacatattcaacggcttcgg-3', and MB probe sequence is 5'-FAM-cgcgtaagctgcaaccagagctacgcg-DABCYL-3', respectively.

Oligonucleotide coding for the first 58- amino acid residues of the outer membrane protein OMP-2a was chemically synthesized by GenScript BioTech Company (Nanjing, China). DNA fragments were purified using Wizard DNA purification Kit (Promega, Madison, USA), then cloned into pUC57-simple vector using TOPO TA cloning kit (Thermo-Fisher, Waltham, MA, USA). After overnight incubation at 4°C, the ligation mixture was transformed into competent Escherichia coli (DH5α) and grown overnight on agar plates containing ampicillin and X-gal. Positive clones were identified using blue-white screening. Five milliliter LB-broth cultures containing single colonies were grown overnight, shaken at 200rpm at 37°C and subsequently purified using the QIA prep Spin Miniprep Kit (Qiagen, Dusseldorf, Germany). Plasmids DNA were dissolved in 50µl TE buffer for sequencing in GenScript BioTech Company (Nanjing, China). The positive plasmid was named pUC-OMP2a.

CFX96™ real-time system (Bio-Rad, Hercules, CA, USA) was used in this study. The PCR cycling was initiated with 5min of denaturation at 95°C, followed by 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 55~65°C and amplification for 30 sec at 72°C. Fluorescence signal was measured at 45°C. PCR solution formula included 0.5 units of DNA Taq polymerase (Takara, Tokyo, Japan), 1µL each primer, 1µL templates, 2µL dNTPs, 2.5µL 10×PCR buffer, 4µL MgCl2, 1µL MB probe and added ddH2O in a total volume of 25µL. PCR products were verified by 1.5% agarose gel electrophoresis. The sequence was validated by sequencing.

The plasmid DNA from different dilutions was used as a template to established a standard curve using this assay for estimation of sensitivity. For this, the pU57-OMP2a plasmid DNA was serially diluted from $10^8$ to $10^2$ copies in descending order by 10 fold dilution. Standard curve was plotted by absolute quantification where 10-fold dilutions were made. Duplicate reactions were set up for each dilution of known DNA along with buffer and negative DNA control.

Specificity was evaluated by incorporating the DNA of clinical isolated *B. melitensis* as positive standard. The nonrelated pathogens such as *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC700603), *Pseudomonas aeruginosa* (ATCC15442), *Enterobacter cloacae* (ATCC13047), *Enterobacter aerogenes* (ATCC49701), *Staphylococcus aureus* (ATCC29213), *Staphylococcus epidermidis* (ATCC12228), *Enterococcus faecium* (ATCC29212), were used as negative controls. The DNAs of all bacteria were extracted and used as a template to evaluate the specificity.

The intra- and inter-assay coefficients of variation were used to assess the precision of each experiment. The intra-assay coefficients of variation (CV) was determined from duplicate values within all runs of the same assay. The inter-assay coefficients of variation were calculated from the mean values of duplicate assays performed in 10 different days.

All samples of individuals with suspicious brucellosis (n= 312) were mainly collected between July 2016 and August 2017 from outpatients and inpatients at the Infectious

Disease Hospital of Xinjiang Uygur Autonomous Region, Urumqi, China who were also residents of the Xinjiang region. These people included were patients with clinical symptoms suggestive of brucellosis such as fever, sweating, arthralgia, myalgia and weakness, and individuals with intimate contact history with individuals who were infected with Brucellaor their family members showed signs of brucellosis. In addition, 10 blood samples of healthy donors were also incorporated in this study as negative control. The blood samples were taken by the qualified nurses or laboratory technicians. Sera were separated and tested within two hours. Brucellosis was diagnosed according to the criterion published by Ministry of Health of the People's Republic of China (WS 269-2007).

DNAs were extracted from blood samples of suspected patients and healthy donors using DNeasy blood kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations, and subjected to MB-qPCR assay. The Rose Plate Bengal Test (RBPT) and Brucella standard agglutination test (SAT) were carried out in order to detect *Brucella* antibodies. RBPT was performed using an antigen from Infectious Disease Institute of Center for Disease Control and Prevention (CDC), Beijing, China. Agglutination was scored as weak positive, positive, strong positive, or very strong positive. All samples were simultaneously subjected to SAT according to the instruction of the manufacturer. Positive reactions were determined using an agglutinoscope, and the titre given indicated the highest dilution in which 50% or more agglutination occurred in the tube.

A minimum of 8ml of blood (for children 1-3ml) was taken through vein puncture and was injected into the culture vials (Peds plusTM/F for children and Plus Aerobic/F for adults, BD BACTECTM, BD company, Durham, SC, USA). And then, the culture vials were loaded to BD BACTECTM/FX Culture System (BD company, USA) and monitored for 7 days before vials with negative results were removed according to the procedures outlined by the Clinical and Laboratory Standards Institute. If a vial gave a positive result, a 100-µl of blood sample was streaked onto sheep-blood agar plates or *Brucella* medium plate (Mast Diagnostics, Bootle, UK). Plates were incubated at 37°C aerobically and in the presence of 5-10% of $CO_2$. Plates were examined every 3 days for growth. Typical and well-isolated *Brucella*-like colony is small, transparent, raised, and convex, with an entire edge and smooth and glistening surface along the streak lines under a microscope after Gram's stain. In addition, bacterial suspension was also prepared and adjusted to 0.5 McBurney unit (about $10^8$ cfu/ml） with 4.5% saline for VITEK 2 Gram-Negative Identification Card (VITEK 2 GN Test Kit). The microorganism was identified by the VITEK 2 Identification System (bioMerieux, Marcy l'Etoile, France) according to biochemical reactions in the card. The result was reported by the software automatically. Data were analyzed with Pearson's chi-square and Fisher's exact tests, using SPSS software version 22. For all analyses, $P< 0.05$ was considered significant.

To obtain standard DNA, a chemically synthesized DNA fragment encoding the first 58-amino acid residues of OMP-2a protein was cloned into pUC57-simple vector by EcoRV site. The sequencing result for the recombinant plasmid was consistent with the predicted sequence, indicating the plasmid harboring positive DNA was successfully constructed. Schematic representation of plasmid construction and the sequencing reporter were shown in supplementary document 1 and 2.

We have found a fully conserved sequence of the gene coding for the first 58-aa of outer membrane protein OMP-2a conserved in five epidemic *Brucella* species ( *B. abortus* , *B. melitensis*, *B. ovis*, *B. suis*, *and B. canis*, respectively ) from GenBank database by DNA alignment. A pair of primers (JPF and JPR, Figure 1B, red font) was designed to amplify a 104bp-DNA fragment from the OMP-2a coding sequence.

It was reported that stem-loop hairpin probes showed better specificity in gene detection compared to linear oligonucleotide probes or double-strains nucleic acid dye (Roberts, Crothers, 1991), and thus presented a higher sensitivity and signal-to-background ratio than TaqMan probes. Shared-stem molecular beacons were also found to yield better performances than TaqMan probes or SYBR Green I (Wang *et al.*, 2005).

To specifically detect PCR amplified products, a molecular beacon (MB) probe was also designed to fulfil guidelines in this study. The probe possesses a sequence matched perfectly to target

DNA, and flanked with self-paired sequence of 5 oligonucleotides to form a stem. The 5' and 3'-end of the constituting sequence was labeled separately with FAM (fluorophore) and DABCYL (quencher) (Figure 1A). A schematic diagram of the MB probe binding to target sequence is shown on Figure 1.

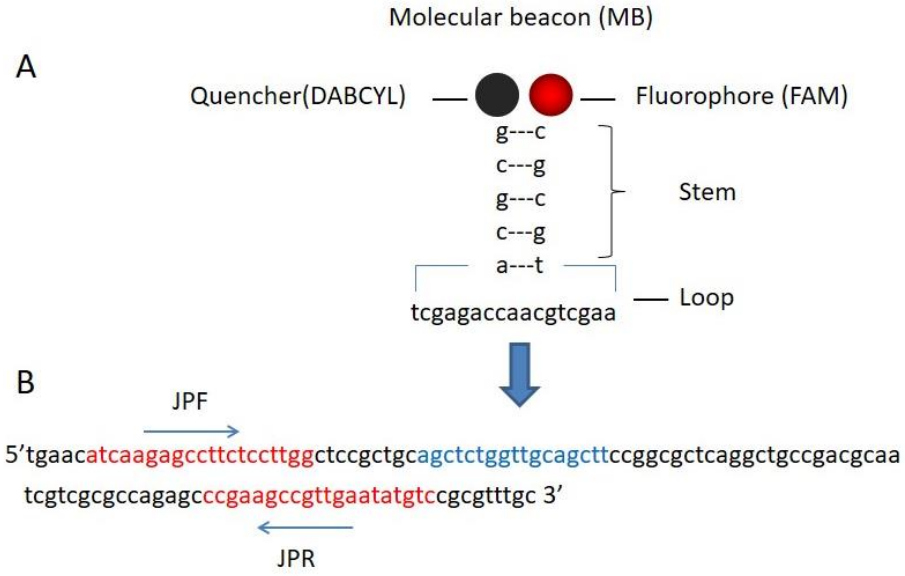


Figure 1. Schematic diagram of the MB probe and its binding to target sequence. A. Constitution and structure of MB probe; B. Oligonucleotide sequence coding for the first 58-aa of outer membrane protein OMP-2a sharing in five epidemic *Brucella* species ( *B. abortus* , *B. melitensis, B. ovis, B. suis, and B. canis*, respectively ). The forward primer (JPF) sequence and the reverse primer (JPR) were indicated as red font; Complementary sequence matched to the probe was indicated as blue font. FAM, carboxyfluoresceine; DABCYL-dimethylaminophenyl-azobenzoic acid.

As predicted, primers JPF and JPR allowed the amplification of a 104-bp fragment at optimal annealing temperature (59°C) from pUC-OPM2a plasmid. A band of about 100bp was observed by 1.5% agarose electrophoresis (Figure 2), which was consistent with the theoretical size. No band was seen using the negative control. The optimal concentration of MB probe and $Mg^{2+}$ was 50pmol/L and 2mmol/L, respectively.

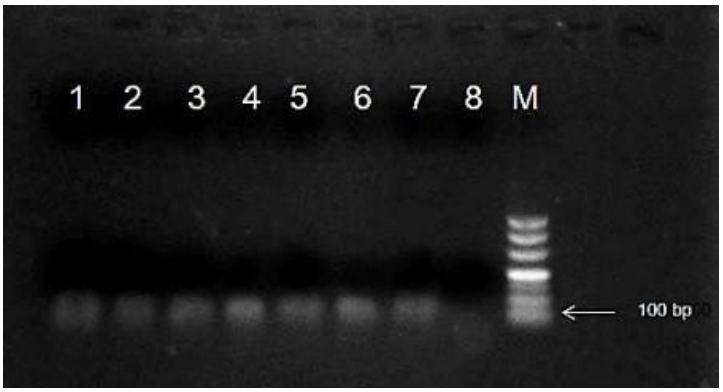


Figure 2. Gel electrophoresis assay of PCR product. Lane1-7, the expected PCR products of about 100bp in 1.5% agarosegel; lane 8, negative control; M, DNA marker.

The sensitivity of this test was studied by using 10-fold serial dilutions of pUC-OPM2a from $10^8$ to $10^2$ copies. The released reporter fluorophore was plotted as a function of the amplification

cycle number. The amplified curve was shown a typical S-type curve (Figure 3A). A standard curve was plotted with cycle threshold (Ct) on the Y-axis against log starting copy number on X-axis (y= -3.1372x+38.201, $R^2$= 0.998.). The standard curve was found linear with a range starting from $10^2$ to $10^8$ copies (Figure 3B). The slope (S) of the linear regression curve correlates with the efficiency (E) of the PCR reaction according to the formula: E = [10–1/slope − 1]. The calculated PCR efficiency for this assay was 98%, based on the slope value of −3.4. It was found that the method could detect *Brucella* DNA as low as 50 copies.

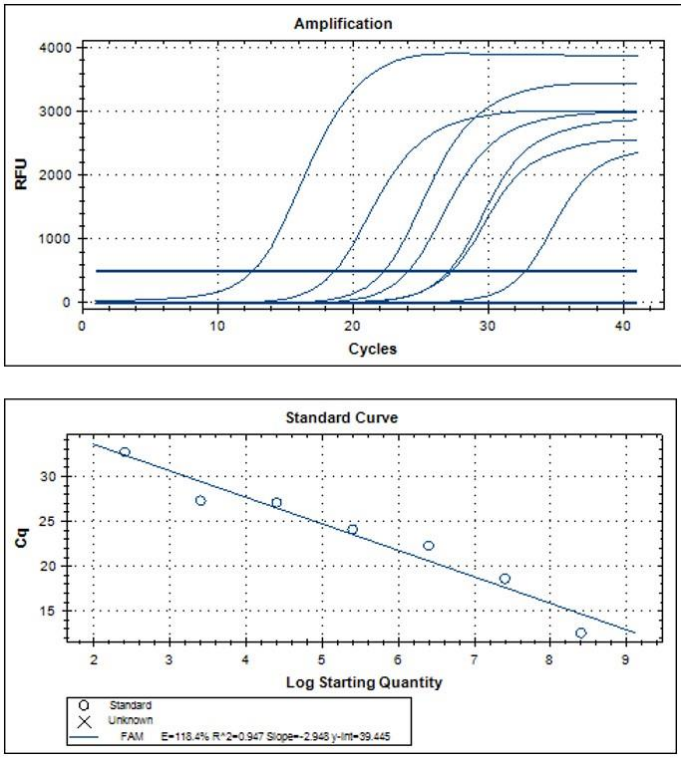


Figure 3. A real-time quantitative PCR assay based on molecular (MB-qPCR). Above, Amplification profiles of 10-fold serially diluted pUC-OPM2a DNA ($10^8$ to $10^2$ copies); below, The standard curve for a 10-fold serial dilution series of plasmid DNA is plotted as the threshold cycle on the Y-axis, against the target concentration (X-axis) (y=-3.1372x+38.201, $R^2$= 0.998). RFU, relative fluorescent Unit.

To evaluate the specificity of the method, and observe the cross-reaction with clinical common bacteria, DNAs extracted from Gram-negative bacteria such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae* and *Enterobacter aerogenes*, or Gram-positive bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis* , *Enterococcus faecium* were used as templates for the PCR assay, while *Brucella* DNA as a positive control. No amplification signal was detected except *Brucella* DNA (Figure not shown). These evidences demonstrated MB-based qPCR was specific for *Brucella*.

The precision was usually evaluated using the intra- and inter-assay coefficients of variation (CVs). The results showed that Ct values had a mean intra-assay CV below 1% and a mean inter-assay CV below 10%. Calculated DNA copy number had mean intra-and inter-assay CVs below 15%, revealing a good reproducibility and stability of the method.

Bacteriological examination was regarded as the "golden standard" method in brucellosis diagnosis, although the bacteriological isolation and identification was a time-consuming procedure. The initial isolation of bacteria was a big problem in diagnostic laboratories (Lealklevezas *et al.*, 1995). Fortunately, the

professional blood-culture vials and culture system had been applied generally in clinical laboratories, the positive percentage of blood culture has dramatically increased in current years. In this study, 108 out of 312 samples were subject to bacteriological examination. The positive samples of blood culture were inoculated onto the plates of sheep-blood agar and *Brucella* agar selective media. After 3-4 days of inoculation, the colonies with pinpoint, glistening, smooth and translucent appearance were subject to Gram's stain and only gram-negative coccobacilli were sent to further confirmatory tests. The results demonstrated that the total positive rate of blood culture was 42.6% (46/108), and bacteria isolated from blood was identified as *Brucella melitensis* biotype 3 by the VITEK 2 Identification System. MB-qPCR exhibited a 97.8% positive rate in positive samples of bacteria culture (45/46). Moreover, *Brucella* DNAs were detected in 13 out of 62 samples which were culture negative (Table 1). The total positive percentage was 53.7%, higher than bacteria culture. The diagnostic sensitivity and specificity was 97.8% and 79.0%, respectively. The positive predictive value, the negative predictive value, and the diagnostic efficiency was 77.6%, 98.0%, and 87.0%, respectively.

Table 1. Test results of MB-qPCR and blood culture (n= 108)

| MB-qPCR | Blood culture | | Total |
|---|---|---|---|
| | Positive (+) | Negative (-) | |
| Positive (+) | 45 | 13 | 58 |
| Negative (-) | 1 | 49 | 50 |
| Total | 46 | 62 | 108 |

To further assess diagnostic value, MB-qPCR should be compared with commercial serological tests such as SAT and RBT which were useful methods for indirect diagnosis or screening of brucellosis. All 312 samples were parallelly determined using MB-qPCR, RBPT, and SAT, respectively. The data were summarized in Table 2 and Table 3. The positive percentage was 47.4% (MB-qPCR), 63.5% (SAT), and 60.7% (RBPT), respectively. There was no statistical difference in positive rates between three methods (P> 0.05). The coincidence percentage of positive results with SAT and RBPT was 91.9%, and 89.2%, respectively. However, the coincidence percentage of negative results was 62.2%, and 59.1%, respectively. Serological tests might reflect that antibody profiles did not have specific clinical correlations, and antibody titers often remained high for a protracted period. Generally, IgM against *Brucella* would appeare as early as the first week after being infected, followed by an IgG amplification at the second week. Both classes of immunoglobulin peaked during the fourth week and persistent for almost one year (Ariza *et al.*, 1992). The antibiotics application was accompanied with a decline in antibody titers. Thus, the positive result of SAT or RBT did not mean present infection, and the false positive/negative results existed obviously (Corbel, 1997, Young, 1995a, 1995b). *Brucella* DNAs were negative in 62 out of 198 samples with SAT positive result, and 67 out of 199 samples with RBPT positive result by MB-qPCR. *Brucella* DNAs were detected in 12 out of 114 samples with SAT negative result, and 16 out of 173 samples with RBPT negative result (Table 2 and Table 3). It was strongly implied that MB-qPCR had a higher specificity and a lower false positive rate than SAT or RBPT.

Table 2. Test results of MB-qPCR and RBPT (n= 312)

| SAT | MB-qPCR | | total |
|---|---|---|---|
| | Positive (+) | Negative (-) | |
| positive (+) | 136 | 62 | 198 |
| negative (-) | 12 | 102 | 114 |
| total | 148 | 164 | 312 |

Table 3. Test results of MB-qPCR and SAT (n= 312)

| RBPT | MB-qPCR | | total |
|---|---|---|---|
| | Positive (+) | Negative (-) | |
| positive (+) | 132 | 67 | 199 |
| negative (-) | 16 | 97 | 173 |
| total | 148 | 164 | 312 |

In this study, we successfully developed a real-time quantitative PCR method based on molecular beacon (MB-qPCR) to detect conserved sequence of the gene coding for the first 58-aa of outer

membrane protein OMP-2a sharing in five epidemic *Brucella* species. It exhibited a high diagnostic sensitivity compared with bacteria culture, and a high specificity compared with serological tests such as SAT and RBPT. It provided an alternative method for molecular diagnosis of brucellosis in the clinicstudy.

Keywords: brucella genome detection, brucellosis diagnosis, molecular beacon, quantitative PCR

**RESUMO**

*O objetivo deste comunicado é desenvolver um método quantitativo PCR em tempo real, baseado em guia molecular (MB) (MB-qPCR) para detecção de infecção por espécies de* Brucella, *e avaliar seu potencial de utilização clínica. Os* primers *e as sondas MB foram desenhados para amplificação específica e determinação de sequência conservada do código do gene para os primeiros 58-aa da proteína de membrana externa OMP-2a, que é compartilhada em cinco espécies de* Brucella *epidêmicas. A avaliação metodológica foi realizada por análise de sensibilidade, especificidade, coeficiente de variação intra e inter, e a linearidade do qPCR. O potencial diagnóstico foi avaliado comparando-se o método qPCR desenvolvido com ensaios de exames bacteriológicos convencionais, incluindo os testes de soroaglutinação convencionais (SATs) e os testes do Rosa Bengala (RBPTs). O método exibiu alta sensibilidade (tão baixo quanto 50 cópias) e grande faixa de linearidade ($10^2$-$10^8$ cópias). Nenhuma reação cruzada foi encontrada com bactéria clínica comum. A sensibilidade diagnóstica foi superior ao exame bacteriológico, e a especificidade diagnóstica foi superior ao SAT ou ao RBPT. Um método MB-qPCR altamente sensível e específico para DNA de* Brucella *foi estabelecido com sucesso, provando ser uma ferramenta útil no diagnóstico molecular de brucelose.*

*Palavras-chave: detecção do genoma* Brucella, *diagnóstico de brucelose, guia molecular, PCR quantitativo*

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