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RESEARCH ARTICLE OPEN ACCESS

Real-time Multiplex PCR Assay for Identification of Human Brucellosis in Morocco and the Differentiation Between *B. melitensis* and *B. abortus*

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Abstract

Brucellosis, a reported zoonotic disease caused by several species of the genus *Brucella*, is endemic to many countries, including Morocco, and poses a major public health challenge. Available studies and national surveillance data highlight its persistent presence and impact, with an incidence rate that is difficult to estimate owing to under-reporting of cases. Human diagnosis is mainly based on serological tests (Rose Bengal and ELISA) without the routine implementation of molecular techniques, which are faster and more specific. To our knowledge, in Morocco, no study has demonstrated the use of polymerase chain reaction (PCR) as a routine diagnostic tool. This study aimed to develop, test, and optimise a multiplex real-time PCR assay capable of detecting all *Brucella* spp. using the conserved *bcsp31* gene to differentiate between *B. melitensis* and *B. abortus*. Using the KoMa plasmid as an internal extraction control, this study assessed the analytical sensitivity of the assay, which showed a detection limit of ≤10 plasmid copies and 5-10 genomic copies per reaction, including its specificity, which reached 100%, with no amplification observed for non-*Brucella* strains. This pilot study evaluated the diagnostic performance of the assay using 30 suspected brucellosis samples. All 17 were confirmed to be positive by ELISA and Rose Bengal tests, whereas 13 were negative. Seventeen positive samples were confirmed by real-time PCR, which showed 100% agreement (95% confidence interval [CI]: 98%). The results showed that all positive human cases studied were caused by *B. melitensis.* This fast and reliable method is a promising tool for monitoring and managing the rapid clinical diagnosis of human brucellosis. However, validation on a larger scale is required.

Keywords: *Brucella* spp., *B. melitensis, B. abortus*, Multiplex PCR, Real-Time, Molecular Diagnostics, Morocco

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INTRODUCTION

Brucellosis is a globally important zoonotic disease caused by various species of the genus *Brucella.*[1] The two main species responsible for human brucellosis are *Brucella abortus* and *Brucella melitensis.*[2] This disease imposes a heavy socioeconomic burden on many countries.[3] These bacteria are transmitted to humans through consumption of contaminated products or occupational exposure.[4] This pathology remains endemic in many countries, including Morocco.[5]

Human brucellosis is frequently underreported because of its non-specific clinical presentation, which ranges from mild symptoms to severe complications, including its variable incubation period and the general lack of awareness regarding the disease.[6-8] Diagnosis cannot be based solely on clinical criteria.[7] Consequently, diagnostic delays are a major challenge, often allowing the infection to progress from an acute to a chronic condition, thereby increasing the risk of complications.[9] Therefore, a rapid and accurate laboratory confirmation is essential.

Isolation of *Brucella* spp. remains the gold standard for diagnosis. However, the culture sensitivity is often low, potentially leading to false-negative results.[10] Furthermore, isolation requires Biosafety Level 3 (BSL-3) facilities and highly skilled personnel owing to the significant risk of laboratory-acquired infections.[3] Alternatively, serological tests detecting lipopolysaccharides (LPS) are widely implemented.[11] However, their specificity is limited by cross-reactivity with other Gram-negative bacteria that share similar antigens, such as *Yersinia enterocolitica.*[7]

To overcome these limitations, molecular methods, particularly qualitative and quantitative polymerase chain reactions (PCR), have been developed to improve diagnostic capabilities.[12-14] Multiplex real-time PCR offers rapid screening and allows for the simultaneous detection of the genus *Brucella* and differentiation between *B. melitensis* and *B. abortus.*[15,16] These methods offer significant advantages in terms of speed and cost-effectiveness compared with traditional screening.[7,15]

In Morocco, accurate incidence estimates are hampered by under-reporting.[17] Diagnosis relies primarily on serological methods despite their limitations, whereas molecular approaches are rarely used in routine protocols.[18] To our knowledge, the routine use of PCR for brucellosis diagnosis has not yet been established in Morocco.

This study's primary objective was to develop and evaluate molecular tools for diagnosing *Brucella* infection. Specifically, this study optimised a multiplex real-time PCR targeting the *bcsp31* sequence for genus-level detection and differentiation of two major species involved in human infection: *B. melitensis* and *B. abortus*. A synthetic plasmid (KoMa) was used as an internal extraction control (IEC) to validate the analytical procedure.

The second objective of this pilot study was to validate the analytical performance of the assay, specifically its specificity and limit of detection (LOD), and evaluate its utility in clinical samples from cases confirmed by ELISA and Rose Bengal assays.

MATERIALS AND METHODS

Sources of strains and DNA

A panel of *Brucella* and non-*Brucella* strains was selected from the American Type Culture Collection (ATCC) Collection and maintained at the Laboratory of Medical Microbiology of Epidemic Diseases, National Institute of Hygiene (Table 1). The genomic DNA of *B. melitensis* and *B. abortus*, used as positive controls, was provided by the Robert Koch Institute (RKI), Berlin.

Evaluation of analytical specificity

Analytical specificity was evaluated using a panel of *Brucella* and non-*Brucella* strains described in Table 1. All the strains were cultured under standard laboratory conditions. Genomic DNA was extracted using a commercial nucleic acid purification kit according to the manufacturer's instructions.

The multiplex real-time PCR assay employed specific primers and hydrolysis (TaqMan) probes targeting *Brucella* spp., with an internal amplification control (IAC) included in each

Table 1. List of bacterial strains used to evaluate assay specificity

Bacterial Strains	Origin (Reference)	No. of Strains
Escherichia coli	ATCC 25922	3
Haemophilus influenzae	ATCC 49247	2
Neisseria meningitidis	ATCC 13090	2
Pseudomonas aeruginosa	ATCC 27853	2
Staphylococcus aureus	TCC 25923	2
Staphylococcus epidermidis	ATCC 12228	2
Streptococcus pyogenes	ATCC 19615	2
Streptococcus pneumoniae	ATCC 49619	2
Enterococcus faecalis	ATCC 51299	2
Listeria monocytogenes	ATCC 7644	2
Shigella sonnei	ATCC 29930	2
Yersinia enterocolitica	ATCC 27729	3
Klebsiella pneumoniae	ATCC 700603	2
Salmonella enteritidis	ATCC 13076	2
Bacillus subtilis	ATCC 6633	2
Candida albicans	ATCC 14053	2
DNA *B. melitensis*	ATCC 23456	2
DNA *B. abortus* S19	NCBI ID 430066	2

*ATCC: American Type Culture Collection *NCBI: National Centre for Biotechnology Information

reaction to monitor PCR inhibition. The specificity was defined as the percentage of non-*Brucella* strains that yielded negative results (without amplification). This was calculated using the following formula:

$$\text{Analytical Specificity (\%)} = \frac{TN}{(TN + FP)} \times 100$$

Where TN represents the number of True Negatives (non-*Brucella* strains with no amplification), and FP represents the number of False Positives (non-*Brucella* strains showing amplification).

Analytical sensitivity assessment

Analytical sensitivity was determined using serial 10-fold dilutions of *Brucella melitensis* genomic DNA, ranging from an initial concentration of 1 ng/µL down to 1 pg/µL. Each dilution was tested in duplicate under the optimised multiplex amplification conditions. A standard calibration curve was generated by plotting the recorded threshold cycle (Ct) values against the logarithm of the initial DNA concentration. The LOD was defined as the lowest DNA concentration consistently detected with a reliability of ≥95% among replicates.

Clinical samples

In the present study, 30 human serum samples were selected from the Biobank of the Medical Laboratory of Epidemic Diseases, National Institute of Hygiene (Rabat, Morocco). The samples were obtained from patients with suspected brucellosis in southern Morocco. Before this study, all samples were characterised using Rose Bengal and ELISA; 17 were confirmed to be positive for brucellosis, and 13 were confirmed to be negative.

Genomic DNA extraction from bacteria and serum

DNA was extracted from 200 µL of serum or bacterial culture using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Five microlitres of a plasmid containing the artificial KoMa2 sequence were added to each sample as an IEC.[19] DNA was eluted in 100 µL of elution buffer. DNA concentration and purity were assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). Extracts were stored at -20 °C until analysis.

Real-time PCR assays

A multiplex TaqMan real-time PCR assay was developed to simultaneously target the

bcsp31 gene (specific to the genus *Brucella*) and the species-specific sequences of *B. abortus* and *B. melitensis*. A separate singleplex assay targeting the KoMa2 sequence was used to monitor the IEC. Primers and probes were designed by the RKI (Berlin, Germany) and synthesised by Qiagen (Alameda, USA); the sequences and concentrations are detailed in Table 2.

PCR reactions were prepared in a final volume of 25 µL, containing 6.25 µL of TaqMan™ Environmental Master Mix (Applied Biosystems), 0.75 µL of each primer, 0.25 µL of each hydrolysis probe, 8.50 µL of nuclease-free water, and 5 µL of template DNA. All samples were assayed in duplicates. Sterile PCR-grade water and purified *Brucella* genomic DNA were used as the negative and positive controls, respectively.

Thermal cycling was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems) with the following protocol: initial activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 60 sec. Data acquisition and Ct calculation were performed using QuantStudio Design & Analysis Software.

Table 2. Primers and oligonucleotide probes used in multiplex real-time PCR for the detection of *Brucella* spp., *B. abortus*, and *B. melitensis* with KoMa2 extraction control[19,20]

Target	Primer/Probe	Sequence (5'-3')
Brucella spp.	*Brucella* spp.-F	GCT CGG TTG CCA ATA TCA ATG C
	Brucella spp.-R	GGG TAA AGC GTC GCC AGA AG
	Brucella spp.-TM	FAM-AAA TCT TCC ACC TTG CCC TTG CCA TCA-BHQ-1
B. abortus	*B. abortus*-F	GCG GCT TTT CTA TCA CGG TAT TC
	B. abortus-R	CAT GCG CTA TCA CGG TAT TC
	B. abortus-TM	HEX-CGC TCA TGC TCG CCA GAC TTC AAT G-BHQ-1
B. melitensis	*B. melitensis*-F	AAC AAG CGG CAC CCC TAA AA
	B. melitensis-R	CAT GCG CTA TGA TCT GGT TAC
	B. melitensis-TM	Cy5-CAG GAG TGT TTC GGC TCA GAA TAA TCC ACA-BHQ-2
KoMa (IEC)	*KoMa*-F	GGTGATGCCGCATTATTACTAGG
	KoMa-R	GGTATTAGCAGTCGCAGGCTT
	KoMa-TM	HEX-TTCTTGCTTGAGGATCTGTCGTGGATCG-BHQ-2

F: Forward primer; R: Reverse primer; TM: TaqMan Probe

Table 3. Multiplex real-time PCR amplification results for *Brucella* controls, non-*Brucella* strains, and clinical serum samples

Sample type	*Brucella* spp. (Target)	*B. melitensis* (Target)	*B. abortus* (Target)
B. melitensis (Control)	+	+	-
B. abortus (Control)	+	-	+
Non-*Brucella* strains	-	-	-
Serum samples (n = 30)	17 (Positive)	17 (Positive)	0 (Positive)

Note: '+': amplification detected; '-': no amplification

Table 4. Agreement between genus-specific multiplex real-time PCR and ELISA for human sera (n = 30)

Method	Cohen's κ	95% Confidence Interval	P-value
Genus-specific PCR vs ELISA	1.00	1.00a-1.00	<0.001

Notes: κ = Cohen's kappa coefficient
Statistical Agreement: The genus-specific multiplex real-time PCR demonstrated perfect agreement with ELISA results (κ = 1.00; P < 0.001) in the studied population

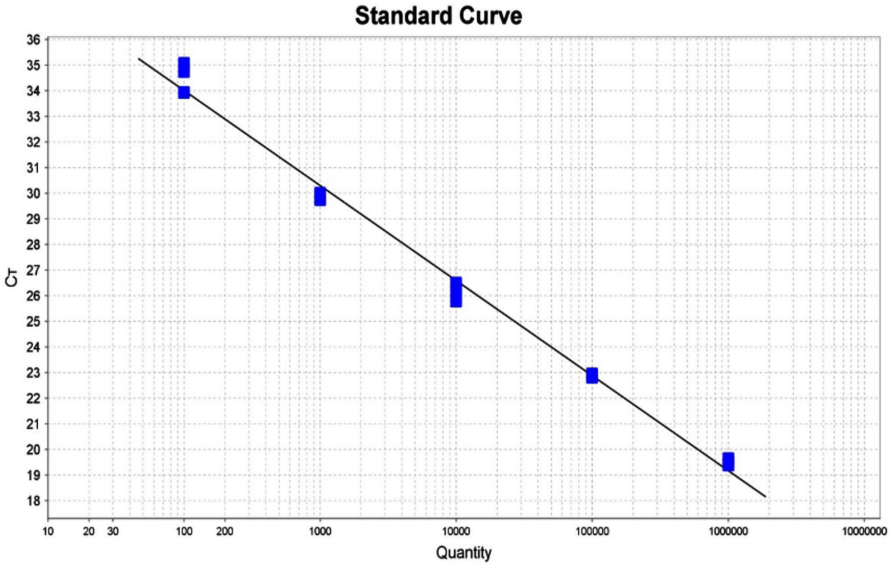

Figure. Standard curve to correlate the DNA concentration of *B. melitensis* in copies/µL with the cycle threshold (Ct)

Table 5. Multiplex real-time PCR detection rates in seropositive serum samples (n = 17)

Sample Group	Test 1 (Positive)	Test 2 (Positive)	Total (Positive) (n)	Total Tested (N)	Detection Rate
Seropositive Sera	17	17	17	17	100%

Note: The assay demonstrated 100% agreement with reference serological results across technical replicates

Internal quality control

Each PCR run included negative (nuclease-free water) and positive controls. An IAC was incorporated into each sample reaction. Plasmid standards were also included to allow for semi-quantitative evaluation and to serve as additional positive controls.

Contamination testing

To assess potential cross-contamination, 6 controls (3 positive and 3 negative) were processed simultaneously during DNA extraction and amplification. These controls were arranged in an alternating pattern on a PCR plate (positive/negative) to monitor the material transfer between wells.

Practicability

The robustness, reproducibility, and clinical performance of the multiplex assay were evaluated through an interlaboratory comparison organised by the RKI. All participating laboratories used the same protocol for *Brucella* genus identification and species differentiation (*B. abortus* and *B. melitensis*). The concordance between centres was used to validate the assay's practicability across different laboratory conditions.

Statistical analysis

Quantitative data (fluorescence signals and Ct values) were generated using the QuantStudio 5 software (Applied Biosystems). Diagnostic accuracy parameters, including sensitivity, specificity, and overall concordance with reference serological assays, were calculated using Microsoft Excel. The results are reported with 95% Confidence Intervals (95% CIs). Cohen's kappa coefficient was calculated to quantify the agreement between the multiplex real-time PCR

assay and the serological methods for the 30 clinical samples evaluated.

RESULTS

Interpretation criteria

The results were interpreted as follows: a sample was considered positive if an amplification curve was detected in the *Brucella* spp. channel with a Cycle Threshold (Ct) <38. A sample was considered negative if no signal was detected in the *Brucella* channel, provided that the IEC (KoMa) had a Ct < 35, confirming valid extraction and amplification. Samples showing no signal for either target were classified as invalid and were retested.

Clinical performance and detection

Among the 30 suspected cases tested in the present cohort, 17 tested positive by multiplex real-time PCR, demonstrating 100% clinical sensitivity and concordance with serological results. All 17 positive samples were identified as *B. melitensis* (Table 3). *B. abortus* DNA was not detected in any of the clinical samples.

The KoMa internal control was successfully detected in all samples (mean Ct: 35 ± 3). The Ct values for the *Brucella*-specific targets ranged from 25-33, indicating moderate to low bacterial loads. The Ct values for *B. melitensis* specific targets ranged from 30-38. No amplification was observed in the 13 seronegative samples or the healthy controls, confirming 100% clinical specificity. In the studied population, the genus-specific multiplex real-time PCR assay showed perfect agreement with ELISA results, with a Cohen's kappa coefficient of 1.00, indicating excellent concordance (Table 4).

Analytical specificity

No cross-reactivity was observed. All *Brucella* strains were correctly detected, whereas none of the non-*Brucella* strains generated specific signals. Consequently, analytical specificity was calculated to be 100%, as shown in Table 3.

Standard curve and LOD

The assay demonstrated high linearity with a correlation coefficient (R^2) of 0.99 and an amplification efficiency of 95%. The LOD was 5-10 genome copies per reaction for *B. melitensis* genomic DNA and 10 copies per reaction for plasmid standards (Figure).

Sample size and concordance

Multiplex real-time PCR successfully detected *Brucella* DNA in all seropositive samples, demonstrating 100% concordance with serological results. These findings are summarised in Table 5.

DISCUSSION

The analytical performance of the multiplex real-time PCR assay has proven to be robust for the routine diagnosis of *Brucella* spp., which is consistent with the findings reported in several studies.[21] Under the conditions of this study, a specificity of 100% excluded cross-reactions with related bacterial species and a LOD of approximately 5-10 genomic copies per reaction allowed for the identification of very low DNA loads, thereby supporting early patient management.

The concordance between multiplex real-time PCR and serological testing is encouraging. However, the rather wide 95% confidence interval (90%-100%) reflects the restricted sample size and indicates high uncertainty around this estimate. Nevertheless, the low detection limit and high specificity underscore the assay's utility as a fast and accurate diagnostic tool.[21] The multiplex real-time PCR developed here demonstrated analytical sensitivity comparable to that of the most reliable methods reported in the literature, and absolute specificity against non-*Brucella* pathogens.[12] Furthermore, inclusion of the KoMa plasmid as an internal control ensured the validity of the DNA extraction and amplification steps, thereby minimising false-negative results.[22]

Human brucellosis is characterised by broad and non-specific clinical manifestations. The clinical presentation alone does not allow for a definitive diagnosis.[23,24] Untimely treatment also increases the risk of chronic complications. Consequently, early and accurate diagnosis is crucial for the treatment, control, and eradication of the disease and serves as the cornerstone of prevention and surveillance in humans.[25] Fast, reliable, sensitive, specific, and automated

detection systems for *Brucella* spp. are urgently needed to facilitate early diagnosis and adequate antibiotic therapy.[2,26]

Considering the limitations of the conventional diagnostic approaches for human brucellosis.[27] In this context, the multiplex real-time PCR assay evaluated in this study provides a rapid, sensitive, and highly specific alternative for detecting and differentiating *Brucella* species.[28] The assay demonstrated excellent clinical performance, full concordance with serological findings, and reliable species-level identification, highlighting its potential utility as a complementary diagnostic tool to support timely diagnosis and improve brucellosis surveillance in endemic regions such as Morocco, where diagnosis currently relies primarily on serological methods.[29,30]

Owing to these constraints, multiplex real-time PCR has emerged as an integrated diagnostic solution that combines speed, safety, and accuracy. Unlike conventional methods, this technique does not require the handling of live agents, thereby reducing the biosafety requirements of BSL-2 for DNA extraction.[31] The optimised protocol provides results in <3 hrs, compared to several days for culture, with an analytical sensitivity of 5-10 genomic copies/reaction.[32,33] The simultaneous detection of specific targets (*bcsp31* for *Brucella* spp., *omp2a* for *B. melitensis*, and *alkB* for *B. abortus*) ensured 100% specificity, as demonstrated in panels that included 20 non-*Brucella* strains.[21,34]

Recent studies increasingly support the added diagnostic value of molecular approaches for brucellosis.[3] Several studies have highlighted the utility of PCR-based methods as confirmatory tools, particularly for complex clinical presentations or when serological results are inconclusive.[35] In endemic settings, molecular assays are recognised as valuable adjuncts to conventional diagnostics, providing improved specificity and aiding in the interpretation of ambiguous serological findings.[36]

Regarding species distribution, *B. melitensis* was identified in 100% of positive cases in this pilot cohort. These observations are consistent with data reported in various publications, indicating that *B. melitensis* is responsible for the majority of human cases.[37,38] Recent publications have noted that routine identification of *Brucella* species involved is often lacking.[39] Currently, data on *Brucella* species circulating in various hosts in Morocco are limited, which hinders the effective study and management of the disease.[36] Identifying the specific species involved in human infections is essential for facilitating epidemiological traceability and applying necessary health measures.[2]

The application of this assay to diverse samples (blood, fixed tissues, and joint fluids) extends its utility to focal forms of the disease that are often culture-negative.[40] In summary, the adoption of multiplex real-time PCR meets modern molecular diagnostic requirements, including speed, safety, and analytical accuracy. Its future integration as a complementary diagnostic approach, consistent with internationally recognised guidelines, could support surveillance and control strategies in endemic regions, such as Morocco.[41]

External quality assessments (EQA) are essential for clinical laboratories to ensure reliable and accurate test results.[42,43] The multiplex real-time PCR test was evaluated through inter-laboratory comparisons, achieving a score of 100%. This guaranteed the effectiveness and sensitivity of the assay for the detection and differentiation of *B. melitensis* and *B. abortus*, as recommended by the World Health Organisation (WHO) for detecting *Brucella* DNA.[44]

Based on the available literature, this study is among the first to use multiplex real-time PCR for the species-specific diagnosis of human brucellosis (*B. melitensis/B. abortus*) in a clinical context in Morocco. The results confirmed that all human cases studied were caused by *B. melitensis*. The assay demonstrated high sensitivity and specificity, allowing for rapid screening and accurate differentiation. However, the limited number of samples prevented extrapolation to national epidemiological trends. The multiplex real-time PCR evaluated in this pilot study is promising for addressing the constraints of conventional methods. However, further multicentre validation involving a larger cohort of clinically suspected cases across diverse geographic regions and varying temporal frames is warranted before its integration into the national diagnostic

algorithm. Future investigations should include additional clinically relevant sample matrices to further confirm the validity of this test.

CONCLUSION

To our knowledge, this is the first report on the molecular diagnosis of human brucellosis caused by *Brucella melitensis* and *B. abortus* in Morocco. The results confirmed that all positive human cases in this cohort were attributable to *B. melitensis*. The developed multiplex real-time PCR assay demonstrated high sensitivity and specificity, offering a reliable complementary tool for the rapid screening and accurate differentiation of the two primary species involved in human infection. The integration of this technique into diagnostic and epidemiological surveillance protocols, particularly within livestock control programs, is recommended as part of an integrated "One Health" approach. Further studies are warranted to improve the understanding of the distribution of *Brucella* species among human and animal populations in Morocco, and to strengthen national control strategies for this persistent zoonosis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AQ, NA, BB, and RC conceptualized the study. AQ, NA, FO, KH, JH, and RC applied methodology. AQ, NA, BB, and RC performed validation. AQ, NA, BB, and RC wrote, reviewed and revised the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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