

Original Research Article

Detection and differentiation of *Brucella abortus* and *Brucella melitensis* by multiplex real-time polymerase chain reaction in patients with pyrexia of unknown origin at a tertiary care centre in Northern India

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ABSTRACT

Background: Brucellosis is a neglected zoonotic disease caused by *Brucella* spp., posing serious public health and economic challenges, especially in low- and middle-income countries. The disease presents with nonspecific symptoms, and conventional diagnostic methods are often time-consuming and less accurate. Multiplex real-time polymerase chain reaction (m-RT PCR) has emerged as a rapid and reliable diagnostic tool for detecting and differentiating *Brucella* species. The prevalence, clinical characteristics, and risk factors of human brucellosis among patients with pyrexia of unknown origin (PUO) and to perform molecular characterization of *Brucella* species using m-RT PCR at King George's Medical University, Lucknow.

Methods: A total of 315 PUO patients were enrolled between January 2023 and March 2025. Blood samples were collected, sera separated, and DNA extracted using the QIAamp DNA Mini Kit. Multiplex RT-PCR assays targeting the *bcs31* and *IS711* genes were performed with species-specific probes to detect *Brucella* spp., *B. abortus*, and *B. melitensis*. Clinical, demographic, and laboratory data were analyzed.

Results: Out of 315 samples, 30 (9.5%) were positive by m-RT PCR-24 (80%) for *B. abortus* and 6 (20%) for *B. melitensis*. Most positive cases were adults (70%) and rural residents (73.3%), with housewives (33.3%) and students (26.7%) being the most affected.

Conclusions: Human brucellosis is endemic in Uttar Pradesh, with *B. abortus* as the predominant species. M-RT-PCR offers a rapid, sensitive, and specific diagnostic method, emphasizing the need for public awareness and a One Health approach for effective control.

Keywords: Brucellosis; Multiplex real-time PCR, *Brucella abortus*, *Brucella melitensis*, Pyrexia of unknown origin

INTRODUCTION

Brucellosis is a zoonotic bacterial infection caused primarily by bacteria of the genus *Brucella*, affecting both humans and a variety of domestic and wild animals.^{1,2} The disease remains a significant public health issue globally, particularly in low- and middle-income countries, despite aggressive control efforts in many regions. Brucellosis is also known as Malta fever, undulant fever, or

Mediterranean fever, and it is transmitted to humans mainly through direct contact with infected animals or consumption of unpasteurized dairy products.^{3,4} The clinical presentation in humans is highly variable and nonspecific, making laboratory confirmation essential for diagnosis.^{5,6}

There are currently more than twelve recognized *Brucella* species, but *B. melitensis*, *B. abortus*, *B. suis*, and, less

commonly, *B. canis* are the primary causes of human disease.² Infection in animals leads to reproductive problems such as abortions, reduced milk yield, and infertility, resulting in substantial economic losses in livestock production. In humans, brucellosis can manifest acutely or as a chronic relapsing illness, often with symptoms like prolonged fever, night sweats, arthralgia, and fatigue.¹⁻³ Complications can include involvement of various organ systems, particularly the musculoskeletal, hepatic, and reproductive systems.^{6,7}

According to global estimates, more than 500,000 new human brucellosis cases are reported annually, but the actual incidence may be significantly underrepresented due to misdiagnosis and underreporting, especially in resource-limited settings.^{1,2} The world health organization classifies brucellosis as one of the world's most neglected zoonotic diseases. Control of brucellosis requires a One Health approach-addressing both the animal reservoirs through vaccination and surveillance, as well as human risk through public education, food safety measures, and improved clinical diagnostics.^{4,5,8}

Due to the intracellular localization of *Brucella* species and their slow growth, conventional microbiological culture methods for diagnosis are time-consuming and pose biohazard risks to laboratory personnel.⁹ Furthermore, serological tests, while widely used, may have limitations such as false positives, variability in sensitivity, and difficulty in distinguishing active infection from past exposure or vaccination.¹⁰ Consequently, molecular diagnostic tools, such as multiplex real-time PCR assays targeting conserved *Brucella* genes, have emerged as valuable methods for rapid, sensitive, and specific detection of *Brucella* DNA in clinical samples, improving the diagnostic yield especially in complicated or focal forms of the disease.⁹⁻¹¹

METHODS

Study setting

This work was carried out in the department of microbiology at King George's Medical University UP, Lucknow.

Inclusion criteria

Patients presenting with fever $\geq 38^{\circ}\text{C}$ ($\geq 100.4^{\circ}\text{F}$) lasting for more than three weeks without an established diagnosis after routine clinical evaluation. Individuals of all age groups and genders, attending outpatient or inpatient departments at King George's medical university, Lucknow. Patients who provided written informed consent to participate in the study were included.

Exclusion criteria

Patients unwilling to provide consent or with insufficient clinical or laboratory data for analysis were excluded.

Sample size

The sample size for this study was 315.

Patients interview and data collection

Using a structured questionnaire all PUO cases that fulfilled the inclusion criteria were enrolled. Demographic details (Name, age, sex of the patient, occupation, address), duration of illness, date of admission, clinical symptoms, and signs, travel history, animal exposure, contact with a patient of similar illness, contact with livestock, handling of the clinical specimen.

Following laboratory findings such as complete blood count, liver function test, kidney function test, random blood sugar, etc. were noted from the record of the patients.

Study population

The study population comprised individuals of all age groups, attending the outpatient and inpatient departments of King George's Medical University, Lucknow with a diagnosis of PUO or suspected with brucellosis.

Sample processing

Patients with PUO were included in the study and tested for brucellosis in the department of microbiology with a 5 mL venous blood sample. The blood samples into serum were separated by centrifugation at 3000 rpm for 3 min and transferred to fresh tubes labeled accordingly. Before processing for further tests, serum samples were stored at 4°C in a freezer, and after serology, samples were stored at -20°C . All details of patients were noted, and relevant history was recorded.

DNA extraction

For nucleic acid extraction from blood samples QIAamp DNA Mini kit (Qiagen, Germany, GmbH) will be used. In brief, 200 μl of the blood sample was added with 20 μl of proteinase K, 200 μl of lysis buffer and incubated at 56°C for 30 min in water bath. After incubation completion, 260 μl of 100% ethanol was added to the lysate. The spin column was then washed and centrifuged followed the manufacturer's directions. Nucleic acid was eluted with 60 μl of elution buffer in fresh recovery tube provided in the kit, after 5 min incubation.

Oligonucleotide primers

For *Brucella* identification, the primers and probe target the bcs31 and IS711 were procured from Eurofins scientific Group, USA (Table 1).²

Oligonucleotide primers and probes will be used in the real-time multiplex PCR assay for the detection of *Brucella* spp., *B. abortus* and *B. melitensis*

Table 1: List of multiplex primer and probes.

Primers	Sequence (5'-3')
<i>Brucella</i> spp. BCSP31 Forward primer	GCTCGGTTGCCAATATCAATGC
<i>Brucella</i> spp. BCSP31 Reverse primer	GGGTAAAGCGTCGCCAGAAG
<i>Brucella</i> spp. BCSP31 RT-PCR probe	AAATCTTCCACCTTGCCCTTGCCATCA-FAM/BHQ1
<i>B. abortus</i> IS711 forward primer	GCGGCTTTTCTATCACGGTATTC
<i>B. abortus</i> IS711 reverse primer	CATGCGCTATGATCTGGTTACG
<i>B. abortus</i> IS711 RT-PCR probe	CGCTCATGCTCGCCAGACTTCAATG HEX/BHQ1
<i>B. melitensis</i> IS711 forward primer	AACAAGCGGCACCCCTAAAA
<i>B. melitensis</i> IS711 reverse primer	CATGCGCTATGATCTGGTTACG
<i>B. melitensis</i> IS711 forward primer	CAGGAGTGTTTCGGCTCAGAATAATCCACA Texas red/BHQ2

Multiplex RT-PCR assay

Multiplex RT-PCR tests were carried in a total volume of 25 µl using Real-time system. PCR reaction plate contained 12.5 µl Takara PCR master mixes (Takara, USA), 0.8µl of each primer (10 pmol/ µl) and probes (5 pmol/ µl) for *Brucella species*, *B.melitensis* and *B.abortus* and 10.1 µl RNase Free H₂O. Thermal cycling conditions was as follows: an initial denaturation at 95 °C for 10 min, followed by 44 cycles at 95° C for 20 s, at 60 ° C for 50 s, and at 72 °for 50 s. Positive and negative controls was used for each experimental study.^{2,3}

RESULTS

A total of 315 serum samples were tested for multiplex RT PCR with reagents procured from Eurofins Scientific Group. Out of the 315 serum samples, m-RT PCR was positive in 30 (9.52%) samples. Figure 2a, showing positive results for *Brucella species*, figure 2b and figure 2c showing positive control of *B. abortus* and *B. melitensis*. Out of 30 multiplex PCR positive samples 24 were found positive for *B. abortus* and 6 were found positive for *B. melitensis*.

Out of 30 multiplex RT PCR cases 21 (70.0%) adults were found positive. Gender wise distribution of multiplex RT PCR, 16 (53.33%), 14 (46.67) were male and female. Ward-wise distribution most of the indoor patients were positive for multiplex RT PCR 28 (93.33%).

Residence-wise most of the patients belonged to the rural area for multiplex RT PCR 22 (73.33%). In occupational distribution, most of the patients were housewives for multiplex RT PCR 10 (33.33%), and students for multiplex RT PCR 8 (26.67%) (Table 2).

Table 3 shows the association between clinical symptoms and brucella positive patients by multiplex RT PCR. Examining the clinical signs, all patients manifested with fever (100%), followed by joints pain, headache, night sweats and chills were other common signs or symptoms.

Table 4 shows association between complication and brucella positive patients by m-RT PCR. Patients were with Joint pain complications for multiplex RT PCR (70.0%).

Laboratory findings

In the current study laboratory investigations such as complete blood count, liver function test, and kidney function test were noted from the record of the patients and respective laboratories. Table 5 shows the findings of *Brucella* positive patients (Table 5).

Table 6 shows that contacts with animals (buffalo, cow, dog, etc.) were the major risk factor noticed in patients for 60.0% in comparison with consumption of animal products (such as uncooked milk/meat) were noticed in patients 46.67%.

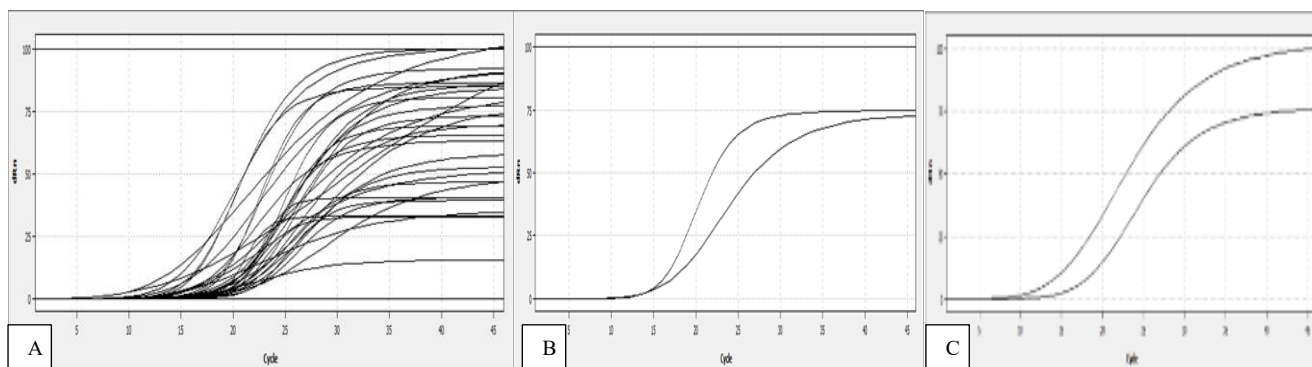


Figure 1 (A-C): A-Multiplex RT PCR graph showing positive result for *Brucella species*. B-Graph showing positive control of *B. abortus* for both genes (BCSP31 and IS711). C-Graph showing positive control of *B. melitensis* for both genes (BCSP31 and IS711).

Table 2: Association between demographical factors and positive cases.

Demographic factors	Multiplex RT PCR, (n=30)	
	N	Percentage (%)
Children (<18 years)	5	16.67
Adults (18-59 years)	21	70.0
Elder (>59 years)	4	13.33
Male	16	53.33
Female	14	46.67
OPD/IPD		
OPD	2	6.67
IPD	28	93.33
Rural	22	73.33
Urban	8	26.67
Housewife	10	33.33
Student	8	26.67
Slaughter house worker	2	6.67
Veterinarian	3	10
Medical staff	2	6.67
Business	1	3.33
Unemployed	3	10
Others	1	3.33

Others*= Private job in software company, painter.

Table 3: Association between clinical features and positive cases.

Clinical features	Multiplex RT PCR, (n=30)	
	N	Percentage (%)
Fever		
Present	30	100.0
Absent	0	0.0
Headache		
Present	15	50
Absent	15	50
Chills		
Present	12	40
Absent	18	60
Night sweats		
Present	14	46.67
Absent	16	53.33
Myalgia		
Present	0.0	0.0
Absent	30	100.0
Joint pain		
Present	21	70
Absent	9	30

Table 4: Association between complication and positive cases.

Complications	Multiplex RT PCR, (n=30)	
	N	Percentage (%)
Joint pain		
Present	21	70
Absent	9	30
Lyphadenopathy (Generalized)		
Present	1	3.33
Absent	29	96.67

Continued.

Complications	Multiplex RT PCR, (n=30)	
	N	Percentage (%)
Respiratory		
Present	0.0	0.0
Absent	30	100
Neurological		
Present	2	6.67
Absent	28	93.33

Table 5: Frequency distribution of laboratory investigations.

Laboratory investigation	Multiplex RT PCR, (n=30)	
	N	Percentage (%)
CBC (Complete blood count)		
HB (Hemoglobin), normal range-13-17	6	20
<13	24	80
TLC- Normal range 4000-11000	20	66.67
>11000	10	33.33
KFT (Kidney function test)		
Serum urea		
Normal range-10-45	23	76.67
>45	7	23.33
Serum creatinine		
Normal range 0.6-1.5	19	63.33
<0.6	2	6.67
>1.5	9	30
LFT (Liver function test)		
SGOT		
Normal range <40 IU/L	11	36.67
>40	19	63.33
SGPT		
Normal range <45	9	30
>45	21	70

Table 6: Association between risk factors and positive cases.

Risk factors	Multiplex RT PCR, (n=30)	
	N	Percentage (%)
Contact with animal		
Yes	18	60
No	12	40
Animal product consumption (Uncooked milk/meat)		
Yes	14	46.67
No	16	53.33

DISCUSSION

Multiplex PCR (mPCR) has emerged as a powerful tool for the detection and differentiation of *Brucella* species, which are significant agents of zoonotic disease worldwide. Given the high genetic similarity among *Brucella* species and the slow, risky, and cumbersome nature of conventional culture and serological tests, mPCR offers rapid, sensitive, and specific alternatives for diagnosis.

The use of genus-specific primers such as the commonly employed B4/B5 (targeting the *bcs*p31 gene) alongside

species-specific primers (e.g., for *B. melitensis* and *B. abortus*) in a multiplex format enables simultaneous detection and discrimination of *Brucella* species in a single assay, reducing time, labor and biosafety risk. For instance, mPCR targeting the *bcs*p31 gene has shown high sensitivity and specificity in clinical samples, such as blood and serum, and is capable of differentiating *B. melitensis* and *B. abortus* using melting curve analysis or amplicon size differences.¹²⁻¹⁵

Challenges remain in PCR assays, especially inhibition from complex sample matrices like whole blood, which contain substances such as EDTA or heparin. In such

cases, the use of serum and optimized DNA extraction protocols (e.g., commercial kits rather than simple boiling) have improved detection rates. Additionally, modified primers with adjusted annealing temperatures and reduced non-specific bindings have enhanced assay specificity and sensitivity.^{16,17}

Comparative studies show that mPCR assays, combined with post-PCR analysis methods like high-resolution melt (HRM) analysis or restriction fragment length polymorphism (RFLP) provide reliable differentiation between *B. abortus* and *B. melitensis* directly from patient samples, often outperforming conventional culture methods in speed and safety.^{18,19}

mPCR has also been successfully applied to non-invasive sample types such as milk and dairy products to monitor food safety, revealing contamination with *Brucella* spp. and other pathogens. Quantitative mPCR further allows estimating microbial loads, instrumental in epidemiological studies and public health risk assessment.²⁰

This study was carried out at King George's Medical University (KGMU), located in Lucknow, Uttar Pradesh, between January 2023 to March 2025. Whole Blood samples were taken from a number of hospital wards. EDTA whole blood samples were used for molecular diagnosis by RT-PCR, targeting *Brucella* specific primers for more specific and sensitive detection.

The purpose of the study was to evaluate the risk factors, clinical characteristics, and prevalence of brucellosis in individuals who had symptoms that were typical of the illness.

Out of 315 cases of PUO patients, the results of this study showed that, 9.5% tested positive by m-RT-PCR, where 24 (80.0%) were found positive for *B. abortus* and 6 (20.0%) for *B. melitensis*. These findings indicate the endemic character of brucellosis in India and are in line with earlier research done in different parts of the nation.

Our study's molecular prevalence, as determined by m-RT-PCR, was 9.5%, which is quite consistent with other research Thakur et al reported a higher molecular prevalence of 12.2%, which could be attributed to their study population consisting exclusively of high-risk individuals, thereby increasing the likelihood of positive findings. In contrast, Kuila et al reported a molecular prevalence of 4.21%, while Rasheduzzaman et al observed a prevalence of 5.45%.²¹⁻²³

Of the thirty PCR-positive cases in our study, *B. abortus* was detected in twenty-four cases (80.0%), while *B. melitensis* was identified in six case (20.0%). This distribution differs significantly from the findings of Dal et al who reported *B. abortus* in only 1.9% of cases and *B. melitensis* in 98.1% and Sanjuan-Jimenez et al brucellosis; 28 (90.3%) of the 31 focal complications of brucellosis.

Piranfar et al multiplex real time PCR showed 108 positive samples (67.5%), including 56% *B. melitensis* and 44% *B. abortus*. Similarly, Barua et al reported a prevalence of 17.64% for *B. melitensis*. These differences may be attributed to regional variations in animal reservoirs and the nature of human-animal contact in different geographical locations.²⁴⁻²⁶

According to our study, human brucellosis is prevalent in various districts of Uttar Pradesh, indicating a significant public health concern. While most earlier studies have focused on brucellosis in animals, our study emphasizes its transmission from animals to humans, particularly in rural and livestock-exposed populations. The findings underscore the urgent need for improved public health education and awareness regarding the clinical symptoms of brucellosis. Early recognition presenting symptoms including joint discomfort, fever, and headache can result in timely medical intervention, accurate diagnosis, and effective treatment. Enhancing awareness will also help in controlling the disease's spread.

Limitations

In Brucellosis, four species of *Brucella* are pathogenic to humans those are *B. abortus*, *B. melitensis*, *B. canis* and *B. suis*; however, in our study, we targeted only two species, *B. abortus* and *B. melitensis*, due to limited funding.

CONCLUSION

In summary, multiplex PCR represents a sensitive, specific, rapid, and safer diagnostic alternative for brucellosis, with capabilities for genus-level and species-level identification. Continuous improvements in primer design, sample preparation, and assay conditions facilitate robust performance, potentially supporting routine clinical and veterinary diagnostics as well as food safety monitoring.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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