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High prevalence of *Coxiella burnetii* coinfection in brucellosis patients: Molecular evidence from an endemic region in Iran

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Abstract

Brucellosis and Q fever are important zoonotic diseases with overlapping clinical manifestations. Their concurrent occurrence is limited in Iran. This study investigated the prevalence and molecular characteristics of *Coxiella burnetii* infection among brucellosis patients in Fars province, Southwest Iran. A total of 152 blood samples were collected from clinically suspected brucellosis patients (68 men and 84 women) with clinical manifestations of fever, chills, and musculoskeletal pain of less than 8 weeks' duration. All patients were confirmed for brucellosis using serological assays and PCR. Subsequently, all samples were evaluated for *C. burnetii* infection using a species-specific PCR assay. Demographic data and potential risk factors were also analyzed. A remarkably high prevalence of *C. burnetii* infection (76.31%) was detected among brucellosis patients. Livestock exposure was significantly associated with coinfection (p=0.036). Phylogenetic analysis showed close similarity to internationally reported strains. All patients received combination therapy with doxycycline and streptomycin, resulting in clinical improvement. All patients received combination therapy with doxycycline and streptomycin, resulting in clinical improvement. This study provides compelling molecular evidence of a high rate of brucellosis and Q fever coinfection in an endemic region, highlighting a potentially underrecognized public health concern. The findings emphasize the need to consider Q fever in the differential diagnosis of brucellosis, particularly in high-risk populations with livestock exposure. Incorporating doxycycline into empirical treatment regimens represents a rational and effective strategy to ensure adequate coverage against both *Brucella* spp. and *C. burnetii* in endemic settings.

Introduction

Zoonotic diseases cause substantial public health concerns due to direct contact with animals and consuming contaminated food or water (1). Brucellosis and coxiellosis are among the most important zoonotic infections presenting with

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nonspecific clinical manifestations such as fever, chills, and malaise (2). Brucellosis, caused by the *Brucella* genus, infects a wide range of domestic and wild animals as well as humans. This disease has always been considered for both economic loss and public health aspects (3). The geographic distribution of brucellosis varies in different countries. However, many parts of the world are considered endemic areas, including Africa, Latin America, Central Asia, the Mediterranean, and the Middle East, such as Iran (4). According to the intensity of the infection, the provinces of Iran are categorized into four types: very high, high, moderate, and low, among which Fars province is denoted as moderate incidence (5). Due to the impairing complication of brucellosis infection, a preliminary diagnosis is essential to fulfil a precise and on-time treatment (4). Coxiellosis, also known as Q fever and caused by *Coxiella burnetii*, is another globally distributed zoonotic infection except New Zealand (6). The disease may appear in acute and chronic forms in humans. The disease presents in acute and chronic forms, ranging from flu-like illness to severe complications such as endocarditis (7). Domestic ruminants, particularly sheep, goats, and cattle, serve as primary reservoirs, and transmission mainly occurs via inhalation of contaminated aerosols. Thus, it mainly affects rural people having close contact with infected animals (8).

There are few studies on coinfection of brucellosis and Q fever in the world (9, 10). Despite the recognized endemicity of both brucellosis and Q fever in Iran, there is a striking lack of molecular-based studies investigating their concurrent occurrence in human populations, particularly in southern regions such as Fars province (11, 12). Moreover, the substantial overlap in clinical manifestations between these two infections poses a serious diagnostic challenge, frequently leading to underdiagnosis or misclassification of Q fever in patients primarily suspected of brucellosis (13). Given their shared transmission routes, common animal reservoirs, and similar clinical presentations, elucidating the prevalence and determinants of coinfection is essential for improving diagnostic accuracy and guiding appropriate therapeutic strategies in endemic areas. To the best of our knowledge, this study represents one of the first molecular investigations assessing the prevalence, associated risk factors, and phylogenetic characteristics of *Coxiella burnetii* among PCR-confirmed brucellosis patients in Southwest Iran. By integrating molecular detection with epidemiological analysis, this study aims to provide novel insights into the hidden burden of Q fever within a well-defined brucellosis cohort.

Materials and Methods

Sample collection and demographic data

Patients diagnosed with brucellosis who were referred to the medical centers of Fasa University of Medical Science, Fasa (latitude: 28° 56' 17.88" N, longitude: 53° 38' 53.52" E), Fars, Iran, during July 2021 to December 2022 were considered for the study. Accordingly, individuals with brucellosis symptoms (such as fever, chill, weakness and fatigue, musculoskeletal pain, and arthralgia) presented in less than 8 weeks, positive serological and PCR tests for brucellosis, and positive epidemiological factors were considered for positive brucellosis. They did not have a previous history of brucellosis. 152 blood samples consisting of 68 men (44.7%) and 84 women (55.2%), were finally collected.

Demographic data and potential risk factors for brucellosis and its coinfection with Q fever, including sex, age, education, occupation, contact with livestock, consumption of dairy products, and knowledge about brucellosis and Q fever, were recorded.

Brucellosis tests

Serological tests

The Rose Bengal test was primarily conducted to screen serum samples suspected of brucellosis, i.e., showing clinical symptoms and epidemiological risk factors of brucellosis (3, 14). The positive samples were subjected to the Wright assay to detect antibody titer, using the Wright agglutination tube kit (Pasteur Institute, Iran). The assay was conducted as per the manufacturer's instructions. Briefly, after 2-fold serial dilution of the sera in phosphate buffer saline (PBS), *Brucella abortus* antigen was added, and the tubes were incubated for 24-48 h at 37 °C. All tubes were finally compared to the positive control. The serum titer ≥ 1:80 was considered positive. Sera with negative Wright test (<1:80 titer) were subsequently assessed for Coombs Wright test, in which serum titer ≥ 1:40 is considered positive. Sera being positive for Wright

and Coombs Wright test were finally assessed for the 2-mercaptoethanol (2-ME) agglutination test using a 2-ME kit (Pasteur Institute, Iran). Sera with a titer ≥ 1:40 were considered positive for the 2-ME test according to the national guideline against brucellosis (15).

DNA extraction and PCR assay

In order to definitively diagnose brucellosis, the samples were subjected to the Polymerase chain reaction (PCR) test. To do so, Genomic DNA was extracted from the serologically positive blood samples using a DNA extraction kit (Cinnagen, Iran) according to the manufacturer's instructions. DNA concentration was subsequently measured by NanoDrop spectrophotometers (Thermo Scientific, Germany) at 260 nm, and the DNA purity was evaluated according to the absorbance at 260/280 nm. PCR assay was performed using primers B4 and B5 targeting *BCSP 31* gene (B4: 5´-TGGCTCGGTTGCCAATATCAA-3´, B5: 5´-CGCGCTTGCCTTTCAGGTCTG-3´) and a program comprised initial denaturation step of 93°C for 5 min followed by 40 cycles of denaturation at 90°C for 1 min, annealing at 30°C for 1 min and extension at 72°C for 1 min, and the final extension at 72°C for 10 min, by a thermocycler (ABI, CA, USA). Amplified products (224 bp) were detected in 1.5% agarose containing 0.5 µg/mL safe stain after electrophoresis (16, 17).

DNA extraction and PCR assay for Q fever

As Q fever, a zoonotic disease, is endemic in this region and has similar clinical symptoms to brucellosis, a PCR assay was performed on the samples to evaluate *C. burnetii* infection simultaneously. To do so, blood samples with serological and PCR-positive brucellosis were subsequently assessed for *C. burnetii* infection using the PCR method. Genomic DNA from the blood samples was extracted using a DNA extraction kit (Cinnagen, Iran) according to the manufacturer's instructions. PCR assay was then carried out using a pair of species-specific *C. burnetii* primers targeted to the *LPS* gene (lipopolysaccharide assembly protein LptD) and designed by the authors using Primer3 Input version 0.4.0 software (18). The primers included forward: 5'-GGACCAGCAACAAAAGAGC-3' and reverse: 5'-CATGGCGCAAGGTGATTACG-3'. After evaluating the purity of extracted DNA, the PCR assay was established in a final reaction volume of 25 µL, containing 5 ng total DNA, 1 pmol of each primer, and Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Denmark). PCR program was executed with an initial denaturation step of 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 sec, annealing at 59.5°C for 20 sec, and extension at 72°C for 10 sec, and the final extension at 72°C for 10 min, by a thermocycler (ABI, CA, USA). To identify the amplified product (395 bp), 1.4% agarose gel containing 0.5 µg/mL safe stain was finally used.

Sequencing and phylogenetic analysis

To confirm the PCR result, five amplicons were randomly selected and sequenced (Macrogen, South Korea) in both strands. The sequences were then analyzed using Geneious Prime 2020.2.5 software. Sequences of the five isolates were subsequently subjected to the National Center for Biotechnology Information (NCBI) (19) and compared to related sequences available in the GenBank library using the Basic Local Alignment Search Tool (BLAST) program. Ultimately, MEGA (version 10.0) was administered to illustrate sequence alignment, the phylogenetic tree, and identity percentage (20).

Statistical analysis

Data were analyzed using SPSS software version 16.0. The Chi-Square statistic test was also used to assess the relationship between the variables and coinfection of brucellosis with Q fever. P-value< 0.05 was considered a statistically significant difference. The logistic regression model was finally used for the independent variables, with $p<0.25$ obtained from the Chi-Square test.

Results

Brucellosis tests and demographic results

The study was conducted on 152 patients diagnosed with brucellosis based on clinical manifestations, serological and PCR (Fig. 1) tests, and epidemiological risk factors of brucellosis. The most prevalence of brucellosis (32.8%) was ranged between 30-50 years old. Other demographic results are detailed in Table 1.

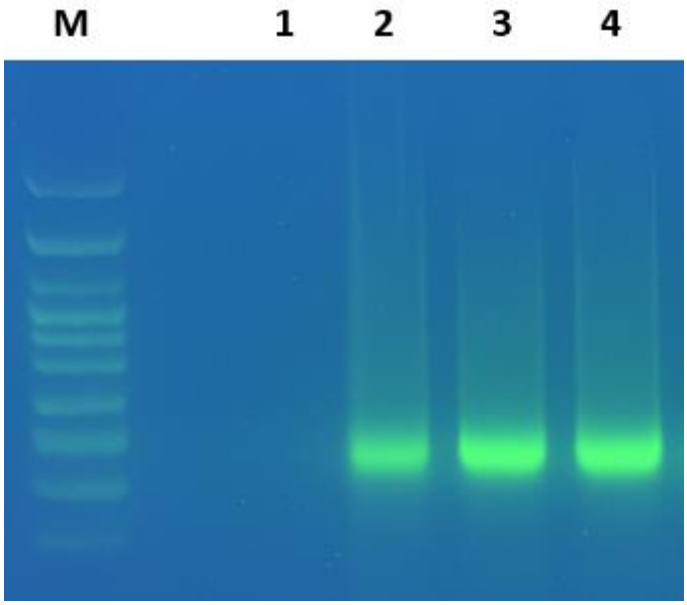

Fig. 1. The gel electrophoresis of PCR products (224 bp) for the *Brucella* spp. *BCSP 31* gene. M: 100 bp DNA marker, Lane 1: Negative control, Lane 2: Positive control, Lanes 3 and 4: Positive samples.

Demographic results of coinfection of brucellosis and Q fever

Demographic results of patients' coinfection with risk factors of the diseases are shown in Table 1. Coinfection was remarkably found with high prevalence in all age groups and educational levels. None of the coinfection-positive patients had any knowledge about Q fever. Chi-Square analysis revealed that there was no relationship between the studied variables (sex, age, education, and consumption of dairy products) and the coinfection occurrence ($p>0.05$). A significant association was observed between coinfection and livestock exposure ($p<0.05$), with ranchers representing the most affected group ($p<0.05$). According to logistic regression analysis, having a history of contact with livestock increased the coinfection with the odds ratio of 2.25 (95% CI: 1.09-4.91, p=0.042).

Q fever PCR

Among 152 brucellosis patients, 116 patients (76.31%, CI: 68.74-82.82) were identified as PCR-positive for Q fever (Fig. 2).

Phylogenetic analysis

The PCR products of the *LPS* gene for five *C. burnetii* isolates were randomly sequenced. The nucleotide sequences were deposited in GenBank using NCBI with the accession numbers of OQ572756, OQ572757, OQ572758, OQ572759, and OQ572760.

Comparing the five sequences against the nucleotide BLAST of the GenBank database elucidated 93.73% identity, 99% Query cover to documented *C. burnetii* sequences. Following the sequence Alignment using MEGA version 10 software, a phylogenetic tree was deduced using the Neighbor-Joining method, was 1000 replicates in the bootstrap test. The whole five sequences are phylogenetically defined in Fig. 3. The phylogenetic tree showed two separate clusters. The isolated fragments distributed in both clusters showed 96.52-100% identity to NCBI nucleotide sequences. The isolate N. 5 showed 96.52% identity with the strain Schperling, an isolated strain from a large outbreak in the Netherlands (21).

Patient treatment

The patients were treated with doxycycline (100 mg/12 h for 6 weeks) and streptomycin (15 mg/kg/day for 3 weeks). After treatment, the patients improved.

Table 1. Demographic characteristics of brucellosis and Q fever patients

Variable		Brucellosis No. (%)	Coinfection with Q fever No. (%)		P-value*
			Yes (%)	No (%)	
Sex	Male	68 (44.7)	51 (75)	17 (25)	0.731
	Female	84 (55.2)	65 (77.38)	19 (22.62)	
Age	< 30	29 (19.08)	23 (79.31)	6 (20.69)	
	31-40	35 (23.02)	27 (75)	9 (25)	0.947
	41-50	50 (32.89)	38 (77.55)	11 (22.45)	
	> 50	38 (25)	28 (73.68)	10 (26.32)	
Education	Illiterate	19 (12.5)	15 (78.95)	4 (21.05)	
	Primary	41 (26.97)	32 (78.05)	9 (21.95)	
	Guidance	43 (28.23)	30 (69.77)	13 (30.23)	
	High school Diploma	30 (19.74)	22 (73.33)	8 (26.67)	0.537
	> High school Diploma	19 (12.5)	17 (89.47)	2 (10.53)	
Occupation	Rancher	95 (62.5)	62 (72.94)	23 (27.06)	
	Student	3 (1.97)	2 (66.67)	1 (33.33)	
	Housewife	17 (11.18)	15 (88.24)	2 (11.76)	**0.025**
	Farmer	38 (25)	30 (78.95)	8 (21.05)	
	Employee	9 (5.92)	7 (77.78)	2 (22.22)	
Contact with livestock	Yes	105 (69)	82(78.10)	23 (21.90)	**0.036**
	No	45 (30.9)	34 (72.34)	13 (27.66)	
Consumption of dairy products	Yes	143 (94.1)	108 (75.52)	35 (24.48)	
	No	9 (5.9)	8 (88.89)	1 (11.11)	0.360
Knowledge about brucellosis	Low	132 (86.84)	103 (78.03)	29 (21.97)	0.201
	Medium	20 (13.16)	13 (65)	7 (35)	
Knowledge about Q fever	Yes	0	0	0	-
	No	152 (100)	116 (76.32)	36 (23.68)	

No.: Number, *P-value of Chi-Square analysis for coinfection

Discussion

Brucellosis and Q fever remain among the most significant zoonotic infections with considerable implications for public health, particularly in endemic regions such as Iran. In the present study, we investigated the prevalence of *Coxiella burnetii* infection among PCR-confirmed brucellosis patients using a molecular approach. The prevalence rate of brucellosis globally varies from less than 1% to as high as 60.0% among high-risk occupations (2). In Iran, seroprevalence studies of brucellosis among high-risk occupations are variously reported from high (43.8% in Lorestan) to low (3.2% in Yazd) prevalence (22, 23).

The remarkably high prevalence of *C. burnetii* infection (76.31%) observed in this study highlights a potentially underrecognized and overlooked public health issue in endemic settings. This prevalence is substantially higher than that reported in previous molecular and seroepidemiological studies in Iran and other regions, suggesting that coinfection may be far more common than currently appreciated (2, 24, 25). Limited information is also available about the brucellosis and Q fever conditions in most parts of Iran (12, 23, 26-28). Q fever seroprevalence varies in different regions of Iran and the world. A systematic review on Q fever seroprevalence from 9 provinces of Iran has elucidated that 19.80% and 32.86% of humans were

respectively positive for chronic and acute Q fever (7). A few studies have been established to identify acute Q fever in suspected patients in Iran. For example, among febrile patients, in Zahedan (southeastern Iran), 35.2%, in Qaemshahr (north of Iran), 5.3%, in Tabriz (northwest Iran) were serologically positive for acute Q fever (29-31). Molecular confirmation of Q fever is also reported on febrile patients in two studies in northwestern Iran, with a prevalence of 4.2% and 7.4% (23, 32).

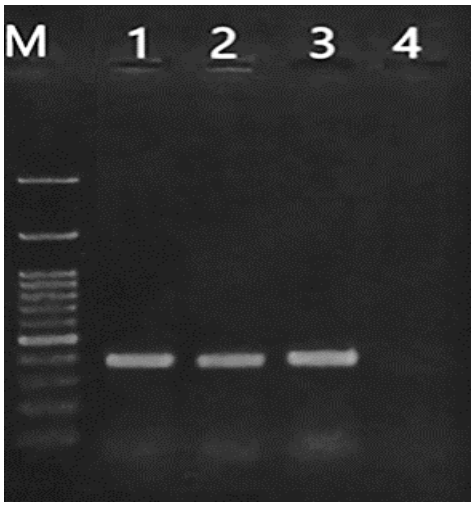

Fig. 2. The gel electrophoresis of PCR products (395 bp) for the *C. burnetii LPS* gene. M: 100 bp DNA marker, Lane 1: Positive control, Lanes 2-3: Positive samples, Lane 4: Negative control

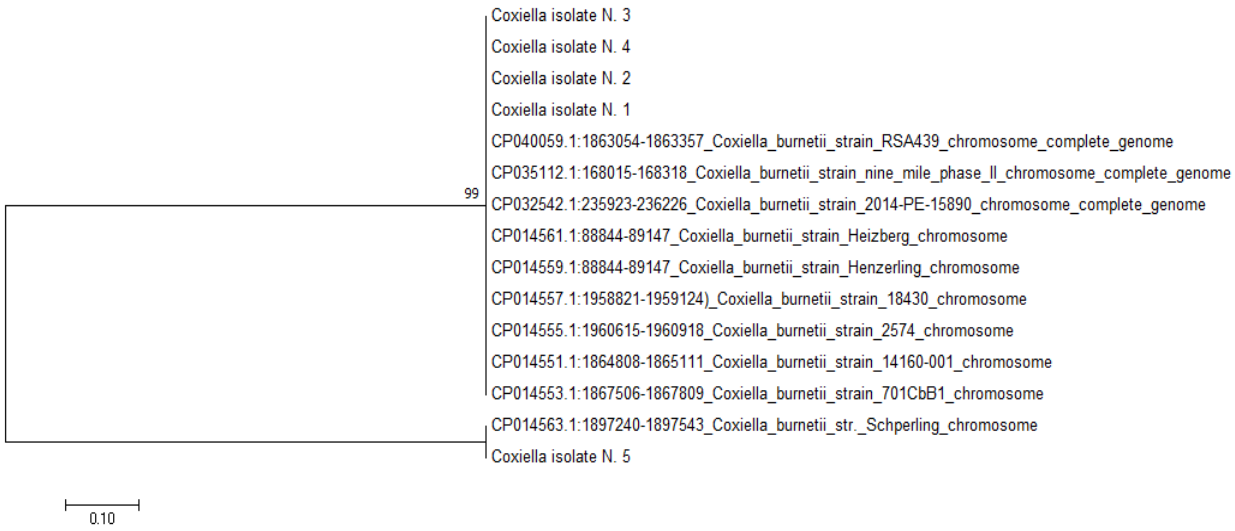

Fig. 3. The phylogenetic analysis from aligned DNA sequences using the Neighbor Joining method. The bootstrap consensus tree was inferred from 1000 replicates.

The variation between our findings and earlier reports may be attributed to differences in study design, geographical conditions, population characteristics, and, importantly, diagnostic methodologies (2). For example, 5% of febrile patients in Northern Tanzania (33), 4.5% in India (34), 0.5% in Thailand (35), and 0.4% in Senegal (36) were positive for acute Q fever; while in a former study in Sokoto State, 44% of the hospitalized patients were *C. burnetii*-positive (37). In Mali, 40% of the patients (38), and in Korea, out of 35 patients with suspected acute Q fever, 46% were recognized as positive (39). Meanwhile, most Q fever reports have been established based on serological assays in Iran or the world, while limited studies have been reported to identify acute or chronic Q fever in patients using PCR. Serological tests have some limitations in diagnosing acute phase coxiellosis; alternatively, molecular tests are rapid and one-step tests that are ideally applicable during the first 2 weeks of infection, before the detectable IgM response (6, 23, 35). Furthermore, PCR test for blood samples can detect the infection if seroconversion has occurred, and is especially important in endemic areas where high levels of background antibodies pose a challenge for serology (27, 40, 41). The use of PCR-based detection in the present study provides strong evidence of active infection. The high rate of coinfection observed in this study can be explained by shared ecological niches, overlapping transmission pathways, and continuous exposure of susceptible populations to infected livestock.

The phylogenetic analysis conducted in this study further strengthens the findings by demonstrating a high degree of genetic similarity between local isolates and internationally reported strains, including those associated with major outbreaks. One of the isolates, in this study, had high identity with the strain Schperling, an isolated strain from a large outbreak in the Netherlands (21). The genome sequence of the Schperling, isolated from human acute Q fever, is found to have high similarity to the Q321 strain, which originated from cattle (21). This suggests possible genetic circulation and epidemiological linkage across regions, emphasizing the need for continuous molecular surveillance.

Q fever remains a neglected and often underdiagnosed disease due to its nonspecific clinical presentation and the high proportion (about 60%) of asymptomatic cases. The symptomatic acute Q fever form is also commonly presented with nonspecific flu-like symptoms, which may be misdiagnosed with other infections (7, 42). Furthermore, in regions where brucellosis is endemic and Q fever is not epidemiologically outlined, the diagnostic focus is often biased toward brucellosis (2). Our findings clearly demonstrate that this diagnostic bias may lead to a substantial underestimation of coinfection rates.

From a clinical standpoint, the coexistence of these infections has significant therapeutic implications. Empirical treatment strategies that focus solely on brucellosis may be insufficient in regions where Q fever is also endemic. For brucellosis treatment, the treatment of brucellosis has historically relied on combination therapy to overcome the intracellular persistence of *Brucella* spp. The World Health Organization and numerous clinical guidelines recommend doxycycline in combination with either streptomycin or gentamicin, or alternatively, with rifampin, for at least six weeks (43-47). The inclusion of doxycycline in treatment regimens is therefore crucial, as it ensures effective coverage against both *Brucella* spp. and *C. burnetii*, ultimately improving patient outcomes and preventing disease progression (48). Notably, other antibiotics used in brucellosis treatment regimens, such as rifampin or aminoglycosides, lack sufficient efficacy against *C. burnetii*. This highlights the therapeutic gap that arises if doxycycline is omitted from empirical therapy in patients living in Q fever–endemic regions. Therefore, the inclusion of doxycycline in first-line regimens for brucellosis treatment ensures optimal efficacy against *Brucella* spp. and *C. burnetii*. This dual coverage is particularly relevant where diagnostic resources are limited, and empirical treatment decisions must be made without laboratory confirmation.

The analysis of risk factors revealed a significant association between coinfection and direct contact with livestock; our result is in accordance with previous studies (3, 7, 23). This finding reinforces the zoonotic nature of these infections and highlights the importance of occupational exposure as a key determinant of disease transmission (5, 49).

Among the most affected groups, rural workers, including farmers, livestock breeders, and shepherds, are the pioneer group affected by brucellosis (49). In endemic rural areas, individuals are frequently exposed to aerosols contaminated with pathogens during animal handling, parturition, or slaughtering processes (50). In contrast, other factors, including sex, age, education, and consumption of dairy products, showed no relationship with the Q fever infection, contrary to some studies (26, 51, 52). Future studies with larger sample sizes with serological methods and multicenter designs are warranted to further elucidate the epidemiology and clinical impact of this coinfection across different regions.

Conclusion

This study demonstrates a high prevalence of *Coxiella burnetii* coinfection among PCR-confirmed brucellosis patients in Southwest Iran, highlighting a potentially underrecognized public health concern in endemic regions. The significant association with livestock exposure underscores the zoonotic nature of transmission. These findings emphasize the importance of considering Q fever in the differential diagnosis of brucellosis and support the inclusion of doxycycline in empirical treatment regimens to ensure effective coverage for both pathogens. Enhanced diagnostic approaches and surveillance are warranted to better address this dual burden.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

The work received ethics approval from the Fasa University of Medical Sciences (IR.FUMS.REC.1398.049). All procedures performed in studies involving human participants were in accordance with the ethical standards of the Declaration of Helsinki. Written and informed consent was obtained from all study participants before the start of the study. For the illiterate participants, Legally Authorized Representatives provided informed consent for the study.

Artificial Intelligence Statement

The authors confirm that there was no use of AI-assisted technology for assisting in the writing of the manuscript.

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