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Blood culture-negative endocarditis caused by *Bartonella quintana* in Iran

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Blood culture-negative endocarditis (BCNE) is a challenging disease because of the significant impact of delayed diagnosis on patients. In this study, excised heart valves and blood serum samples were collected from 50 BCNE patients in two central hospitals in Tehran, Iran. Sera were tested by IFA for the presence of IgG and IgM antibodies against *Bartonella quintana* and *B. henselae*. Genomic DNA extracted from the heart valves was examined for *Bartonella*-specific *ssrA* gene in a probe-based method real-time PCR assay. Any positive sample was Sanger sequenced. IgG titer higher than 1024 was observed in only one patient and all 50 patients tested negative for *Bartonella* IgM. By real-time PCR, the *ssrA* gene was detected in the valve of one patient which was further confirmed to be *B. quintana*. *Bartonella*-like structures were observed in transmission electron microscopy images of that patient. We present for the first time the involvement of *Bartonella* in BCNE in Iran. Future research on at-risk populations, as well as domestic and wild mammals as potential reservoirs, is recommended.

Keywords BCNE, *Bartonella quintana*, IFA, Probe, Real-time PCR, Infective endocarditis, TEM

Abbreviations

BCNE	Blood culture-negative endocarditis
IFA	Indirect fluorescent antibody
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MDC	Modified duke criteria
TEM	Transmission electron microscopy
LV	Left ventricle
RV	Right ventricle
LA	Left atrium
RA	Right atrium
MV	Mitral valve
RCC	Right coronary cusp
DMSO	Dimethyl sulfoxide

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Infective endocarditis (IE) affects the internal structures of the heart[1]. The heterogeneity in etiology and clinical manifestations along with a 30% mortality rate make IE a life-threatening disease despite its low incidence[2]. The diagnosis of IE primarily relies on blood cultures, as outlined in the modified Duke criteria (MDC), which are essential for guiding appropriate antimicrobial therapy and surgical interventions. The situation becomes more complicated when the blood culture, a major criterion in the MDC for the diagnosis of IE fails[3]. In this context, blood culture-negative endocarditis (BCNE) presents a significant challenge for early diagnosis[4,5].

BCNE can be caused by infection with fastidious organisms such as nutritionally variant streptococci, fastidious Gram-negative bacilli of the HACEK group (*Aggregatibacter aphrophilus*, formerly known as *Haemophilus aphrophilus* and *H. paraphrophilus*; *Aggregatibacter actinomycetemcomitans*, formerly known as *Actinobacillus actinomycetemcomitans*; *Cardiobacterium hominis*; *Eikenella corrodens*; *Kingella kingae*), *Brucella* spp. (in endemic areas), and fungi. It can also be caused by infections with intracellular bacteria such as the zoonotic agents *Coxiella burnetii* and *Bartonella*, as well as *Tropheryma whipplei*[6–8]. In the genus *Bartonella*, *B. henselae* and *B. quintana* are the most common species associated with BCNE. However, other species like *B. washoensis*, *B. elizabethae*, *B. alsatica*, *B. koehlerae*, and *B. vinsonii subsp. berkhoffii* also play a role in BCNE[6,7,9,10]. *Bartonella quintana* infections are more likely to spread in communities affected by war, poverty, and famine[11].

The diagnosis of BCNE caused by *Bartonella* species remains challenging, as the bacteria are notoriously fastidious and difficult to culture in standard laboratory conditions. Serological testing, such as indirect immunofluorescence assay (IFA), has proven useful in identifying *Bartonella* infections, but cross-reactivity with other pathogens and low antibody titers in chronic infections can lead to false negatives. Recent advancements in molecular techniques, such as real-time PCR targeting specific genes like *ssrA*, have significantly enhanced the accuracy and efficiency of diagnostics for the *Bartonella* genus[12].

Homelessness and alcoholism are also significant contributing factors. The human body louse (*Pediculus humanus corporis*) is known to transmit *B. quintana*, while *B. henselae* is primarily transmitted by cat fleas e.g. *Ctenocephalides felis*[13–15]. Previous studies reported the prevalence of *Bartonella* species in cats, dogs, and their ectoparasites in different regions of Iran[11,16–18]. However, there was limited information about the possible role of *Bartonella* species in BCNEs[19]. Hence, this study aimed to explore this issue through serology, PCR, and transmission electron microscopy.

Methods

Samples

Between March 2019 and September 2021, fifty excised cardiac valves and their corresponding whole blood samples were collected from patients diagnosed with BCNE who underwent valve replacement surgery at Imam Khomeini Hospital and Shahid Rajaei Heart Center in Tehran. Patients' demographic, clinical, and laboratory data including hematology and biochemistry results, also echocardiograms were collected. A portion of the valve tissues were fixed in a solution of 2.5% glutaraldehyde buffered at pH 7.2 (at room temperature) for 3 h and postfixed in 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer for one hour[20]. The other portion of valve tissue and blood sera were stored in the freezer until further testing.

Serologic testing

The serum obtained from patients' blood samples was utilized in an IFA to identify *Bartonella* antibodies. We used commercial IFA-IgM and IFA-IgG kits (Vircell, Granada, Spain) for the detection of *B. henselae* and *B. quintana* antibodies. The presence of apple-green bacilli was observed under an Olympus BX51 fluorescence microscope (Tokyo, Japan). Titers of 64 to 256 suggest a possible *Bartonella* infection, while titers of 512, combined with clinical symptoms, strongly indicate the disease, as recommended by the kit manufacturer (Vircell, Granada, Spain).

Molecular assays

Genomic DNA of excised valves was extracted using DNP™ kit (SinaClon, Tehran, Iran) and tested using TaqMan probe real-time PCR (qPCR) assay targeting the *ssrA* gene in CFX96 Touch Real-Time PCR Detection System (BioRad™, Milan, Italy)[12].

The *ssrA* qPCR positive sample/s was/were further tested using additional conventional PCR (cPCR) assays that amplify *ssr*A (using the same sense and antisense primers used in cPCR assay) and five extra housekeeping genes including ITS (16S–23S rRNA intergenic transcribed spacer), *gltA* (citrate synthase), *nlpD* (lipoprotein, outer membrane protein), *rpoB* (RNA polymerase ß-subunit), and *bqtR* (*B. quintana* transcriptional regulator). Selected genes are essential for cellular function and ideal for phylogenetic studies. They also include species-specific regions for differentiation among closely related *Bartonella* species. These genes have been validated for *Bartonella* identification and phylogeny, ensuring robust and comparable results[12,21–23].

DNA amplicons were purified using the NEB Exo-SAP PCR purification kit (New England Biolabs, Inc., Ipswich, MA, U.S.A.) prior Sanger sequencing analysis performed in Eurofins Genomics Center (Vimodrone, Italy), using the same primers used in cPCR assays, and analyzed phylogenetically to enhance the phylogeny's ability to distinguish between species, using BioNumerics ver 7.1. (Applied Maths NV, Sint-Martens-Latem, Belgium). For multiple sequence alignment, the sequences of the targeted genes (*ssrA, gltA, nlpD, rpoB, bqtR, ITS*) obtained in this study were combined with reference sequences of *Bartonella* strains retrieved from the NCBI GenBank database. We performed standard pairwise alignment and employed single linkage clustering for the phylogenetic analysis[22,24].

B. henselae str. Berlin-I, *B. henselae* Houston-I, and *B. henselae* Marseille strains that are significant causative agents of BCNE, *B. quintana* strain Toulouse, *B. quintana* strain MF1-1, *B. quintana* strain RM-11, *B. taylorii* of murine origin, and *B. vinsonii* subsp. *berkhoffii* str. NCTC12905, which is agent of endocarditis in dogs and

various complications in humans, and is endemic in different dog populations in Iran[16], were used as positive controls in qPCR and cPCR assays.

Transmission electron microscopy (TEM)

The heart valve samples that exhibited a positive result for *Bartonella* detection using the real-time PCR assay were chosen for examination in a transmission electron microscope. To increase the likelihood of observing bacteria by TEM microscopy, vegetative lesion-containing portions of the valve were selected. The fixed tissue samples were then dehydrated by dehydrated for 15–30 min in ascending ethanol concentrations of 30%, 50%, 70%, 80%, 90%, and 100%. Ultrathin sections of valve tissue (50–70 nm) were stained with 2% uranyl acetate and 0.1% lead citrate to visualize the bacteria in a Philips EM 208S microscope (Royal Dutch Philips Electronics Ltd., Eindhoven, The Netherlands)[20].

Bacterial culture

Following lysis centrifugation, the whole blood samples of all patients under study were inoculated into Colombia agar base supplemented with 5% defibrinated sheep blood (CBA). The plates were subsequently incubated for a minimum of 21 days at 37 °C in a 5% CO_2 atmosphere[25,26].

Results

Fifty patients (41 males, 9 females) underwent replacement of aortic ($n=19$), mitral ($n=19$) and tricuspid ($n=12$) valves. Patients aged 41 years on average (range 3–75 years). None of the patients had a history of drug/alcohol addiction or homelessness. Fever ≥ 38 °C was the most frequently recorded clinical manifestation.

Only one serum exhibited an IgG titer exceeding 1:1024 for both *B. henselae* and *B. quintana*. None of the 50 patients had detectable *Bartonella* IgM antibodies. The seropositive patient was a 55-year-old unemployed man living in Tehran. He had no history of homelessness, alcoholism, valve replacement surgery, or lice infestation.

Blood parameters of that patient were either out of the normal range or critical for clinical decision-making (Table 1).

In pre-surgery transesophageal echocardiography (TEE), the left ventricle was severely enlarged with moderate systolic dysfunction, the right ventricle was moderately enlarged with moderate to severe systolic dysfunction, the left atrium was mildly enlarged, the right atrium was enlarged, and mitral valve leaflets were thickened and tethered. No mitral stenosis was observed, but mild to moderate mitral regurgitation was identified. The aortic valve leaflets were thickened and damaged, resulting in mild aortic stenosis and severe aortic regurgitation. A 27 × 13 mm mobile mass was discovered on the right coronary cusp (RCC), suggesting the presence of vegetation (Fig. 1). The tricuspid valve leaflets were tethered, and although there was no tricuspid stenosis, there was severe tricuspid regurgitation. The pulmonary valve leaflets were normal with moderate pulmonic regurgitation and no pulmonic stenosis. A mobile mass measuring approximately 6 × 3.8 mm was connected to the ventricular side of the pulmonary valve, indicating the presence of vegetation.

Valve genomic DNA from that patient scored positive by qPCR, and the nucleotide sequence of *ssrA* gene was suggestive of *B. quintana*. Amplicons of the expected sizes were obtained also for other targets, as follows. The sample from patient No.10 showed the highest similarity with *B. quintana* MF1-1 strain for the *gltA* gene (100%) and the lowest similarity with *B. quintana* Toulouse strain for the ITS region f (99.4%). Conversely, a low degree of similarity was displayed with the *glt*A gene sequences of *B. henselae* strains (89.7%) and *B. vinsonii* subsp. *berkhoffii* (87.6%) (Fig. 2). Furthermore, *Bartonella*-like organisms organized in the form of invasomes were observed in the TEM micrographs of the patient's aortic valve (Fig. 3).

Discussion

We demonstrated that *B. quintana* also causes BCNE in Iran. A recent systematic review that analyzed cases of *B. quintana* endocarditis reported between 1993 and 2022, and originated from 40 countries, on all continents

Test	Value	Unit	Normal range
WBC	12.8	× 1000/mm^3	(4.1–10.1)
ESR	74	mm/h	Male 51–85 Years < 30
AST	337	IU/L	Adult female < 31 Adult male < 37
ALT	548	IU/L	Adult female < 31 Adult male < 41
ALP	268	IU/L	Adult 70–306
BilT	3.7	mg/dL	Adult 0.1–1.2
BilD	3.6	mg/dL	< 0.3
Urea	51	mg/dL	Adult 15–50
Creatinine	1.7	mg/dL	Adult 0.7–1.4
HBSAg	Non-reactive		–
Anti-HCV	Non-reactive		–
HIV Ag/Ab	Non-reactive		–

Table 1. Laboratory findings for the *Bartonella*-positive patient.

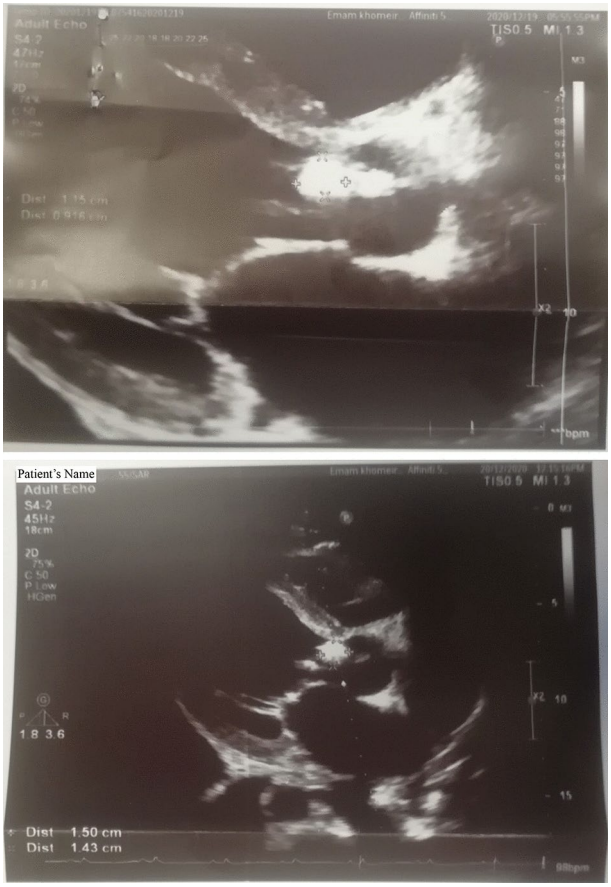

Fig. 1. Echocardiogram of *Bartonella quintana* positive patient. The vegetative mobile mass on the aortic valve is indicated by "+" sign.

except Antarctica, indicated that *B. quintana* may be more common than previously suggested[27]. For instance, in the African continent, the presence of *B. quintana* human infection and lice positivity indicates a hidden burden of illness[28]. It should be noted that *B. quintana* infection and bacteremia can be chronic in humans, as is the case with *B. henselae* infection in cats[29], and that in apparently healthy people, chronic bacteremia and low antibody titers may develop[30]. *B. quintana* infection has been classified by the World Health Organization (WHO) as a neglected tropical disease[31] and that Institution recommended large studies on the prevalence of *B. quintana* infection among individuals presenting with fever, heart failure, or symptoms of embolization for a better understanding of the disease epidemiology.

Negative blood cultures are observed in 2.5–31% of cases of infectious endocarditis in developed nations[32], whereas they characterize 48–56% of cases in developing countries like Pakistan[33], Algeria[34], and South Africa[35]. In a Tunisian study, eleven of thirteen *Bartonella* seropositive samples of BCNE were confirmed as positive using a nested real-time method that targeted the *fur* gene[36]. Another investigation conducted in the United Kingdom identified 12 instances of *B. quintana*, and one instance of *B. henselae* among 14 seropositive samples. This was accomplished through semi-nested PCR, which targeted a fragment of the *16S/23S rDNA* intergenic spacer region[37]. In a 12-year survey on bartonellosis in BCNE cases in Spain, 13 cases were caused by *B. henselae,* and 8 cases were associated with *B. quintana*[38]. In the only previous Iranian report on BCNE cases, five out of 59 patients (8.5%) scored real-time PCR positive which were further typed as *B. quintana*[39]. Therefore, our results confirm *B. quintana* as a main source of *Bartonella* endocarditis in Iran.

The issue of cross-reactivity for *Bartonella* IFA serology assays with *Treponema pallidum*, *Coxiella burnetii*, *Chlamydia* spp., *Rickettsia* spp., *Orientia tsutsugamushi*, *Francisella tularensis*, *Ehrlichia chaffeensis*, *Mycoplasma pneumoniae*, and *Escherichia coli* has been reported[40,41]. Furthermore, reports of cross-reactivity between *B. henselae* and *B. quintana* have been recorded at the species level[19,42,43]. The same phenomenon was observed for *B. henselae* and *B. quintana* in this research. The presence of IgG with the absence of IgM antibodies in the serum sample of positive patients may indicate that the individual was in a chronic infection[44]. Given the substantial postoperative mortality rates observed in patients who have undergone valvular repair/replacement[5] and the invasive nature of *Bartonella* endocarditis, which often results in valve destruction[45,46], as well as the challenges related to cross-reactivity in serological tests, it is advisable to confirm serological results by using the probe-based real-time PCR method, which has excellent sensitivity and specificity for the diagnosis of BCNEs.

Laboratory abnormalities that are frequently detected in cases of *Bartonella* endocarditis encompass the following: increased erythrocyte sedimentation rate, anemia, thrombocytopenia, elevated liver enzymes, renal

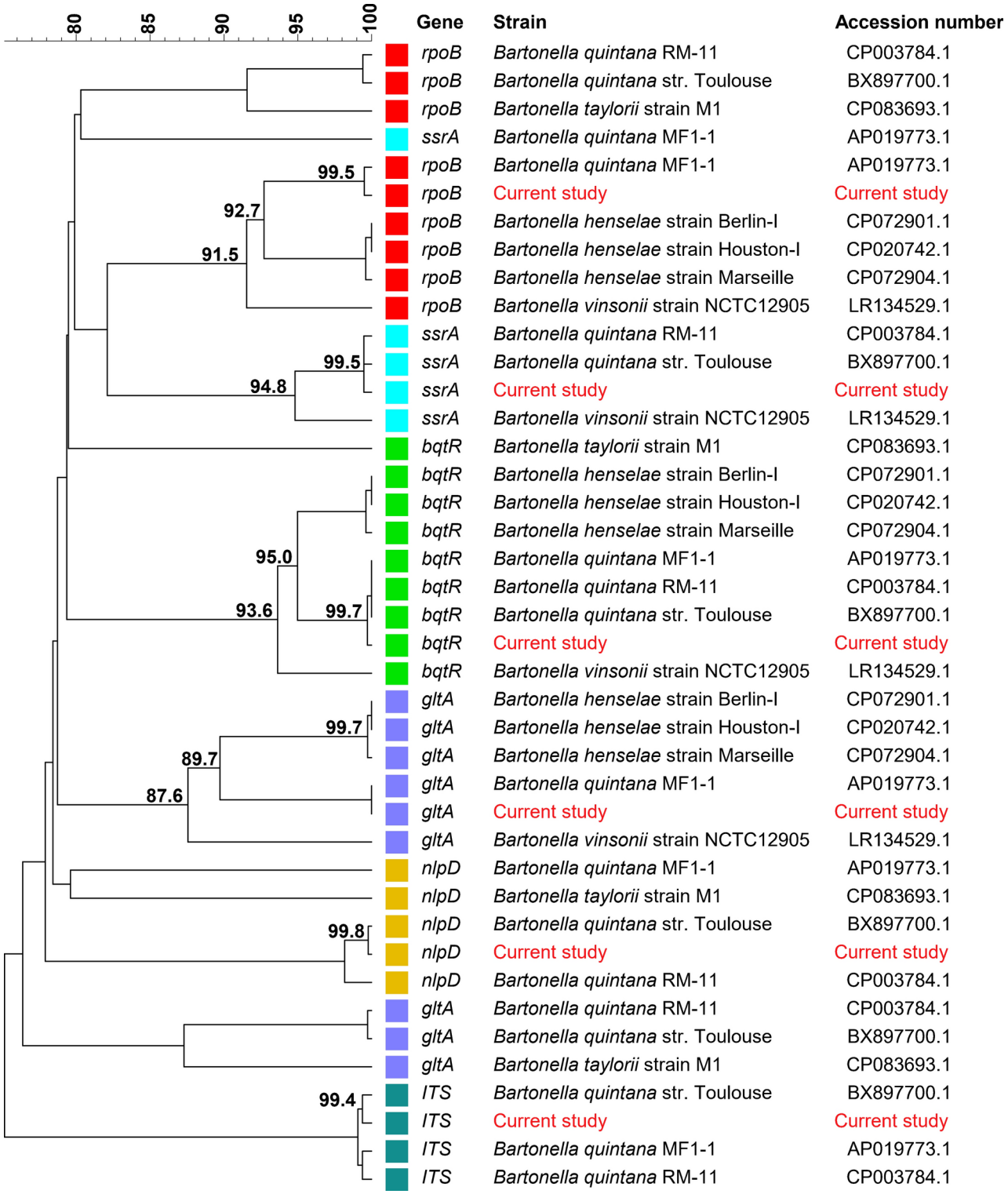

Fig. 2. Similarity dendrogram was constructed using standard pairwise alignment and single linkage cluster analysis for sequences of *ssrA*, ITS, *gltA*, *nlpD*, *rpoB*, and *bqtR*. *nlpD* gene was absent in *Bartonella henselae* strain *Berlin-I*, *Bartonella henselae* strain *Houston-I*, *Bartonella henselae* strain *Marseille*, and *Bartonella vinsonii* strain *NCTC12905* in the GeneBank database. *ssrA* gene was absent in *Bartonella henselae* strain *Berlin-I*, *Bartonella henselae* strain *Houston-I*, *Bartonella henselae* strain *Marseille*, and *Bartonella taylorii* strain *M1* in the GeneBank database. ITS locus was present in only *Bartonella quintana MF1-1*, *Bartonella quintana* strain *RM-11* and *Bartonella quintana* strain *Toulouse* in the GeneBank database.

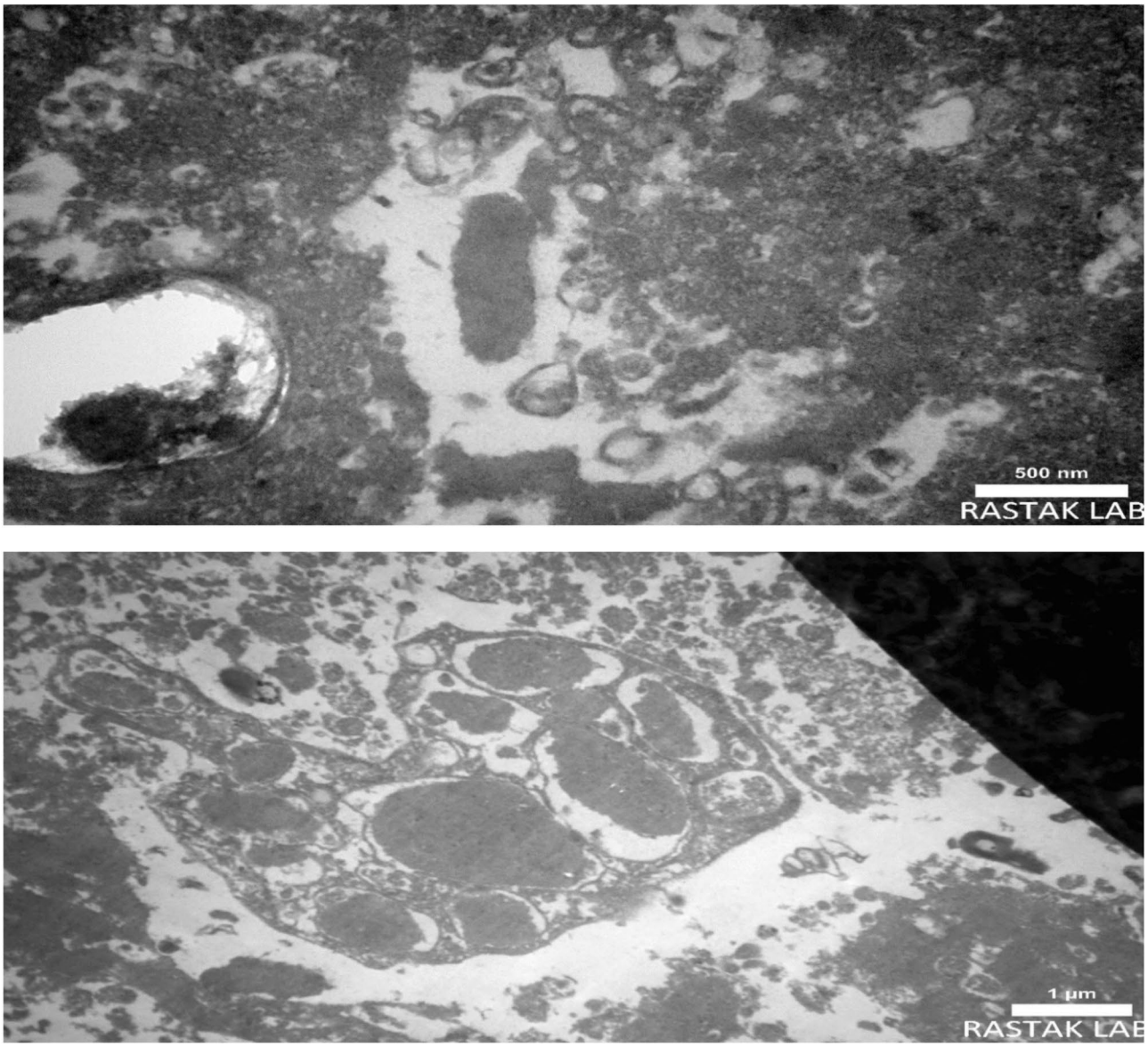

Fig. 3. Transmission electron microscope images of the aortic valve from *Bartonella quintana* positive patient. A cluster of polymorphic *B. quintana* was found in the section prepared from the vegetative mass. Scale bar = 500 nm (up), 1 µm (below).

failure indications, leukocytosis, and a positive rheumatoid factor[26]. Significantly increased levels of BilT and BilD, along with elevated levels of AST, ALT, and ALP hepatic enzymes, indicated the patient's liver alterations as reported in Table 1. The patient's laboratory findings, which showed leukocytosis and an elevated erythrocyte sedimentation rate, suggested the presence of an inflammatory disease. However, these findings are not exclusive to *Bartonella* spp.-caused BCNEs. Glomerulonephritis, an additional complication identified in BCNEs caused by *Bartonella* spp., is further supported by the elevated levels of urea and creatinine[47].

Several genes have been used for molecular diagnosis of the *Bartonella* genus, with the *rpoB*, the *gltA*[23], and the *ssrA*[12] being the most frequently employed. Two significant benefits are associated with the *ssrA* gene that can be utilized in molecular testing of human clinical specimens. Predominantly specific to prokaryotes, tmRNA prevents cross-reactivity with human genomic DNA. Furthermore, the sequence diversity of *ssrA* is adequate to distinguish various human-infective *Bartonella* species[12]. A similar methodology and findings have been reported in a case of *B. quintana* endocarditis in Turkey, where molecular techniques identified the pathogen in the aortic valve[48]. The ITS (16S/23S rRNA intergenic spacer region) sequencing is a valuable tool for distinguishing specific *Bartonella* species, such as *B. henselae*, *B. clarridgeiae*, and *B. bacilliformis*, due to the unique ITS sequences associated with each species.[49]. The *ssrA* gene also shows sufficient sequence variability for genotyping and differentiating strains within the same species. In a comparative study, the sensitivity of *ssrA* and *rpoB* in detecting *Bartonella* was compared[50]. The results showed that real-time PCR targeting the *ssrA* gene was more sensitive than *rpoB*-PCR in detecting *B. clarridgeiae* and *B. quintana* DNA in heart valve specimens.

Furthermore, targeting the *ssrA* gene improved the sensitivity of detecting *B. henselae* in blood samples[50]. The reliability of *ssrA*-based phylogenies is supported by the consistent alignment of phylogenetic relationships obtained from *ssrA* sequences with those derived from other frequently used markers such as *gltA* and *16S rRNA*[12,51]. The *ssrA*-based phylogeny, similar to other single-gene phylogenies, may not provide a comprehensive representation of the entire evolutionary lineage of the genus because of variations in evolutionary rates and gene histories[7,52]. Additionally, researchers have noted instances of recombination occurring within a single gene (*gltA*), which can complicate phylogenetic analysis and may not be found in other sequenced genetic loci[52,53]. The dendrogram in our study, constructed using sequence data, offers higher resolution compared to traditional methods, allowing the detection of subtle genetic variations. For example, the *ssrA* gene in *B. quintana* MF1-1 shares about 80% similarity with other *B. quintana* strains. This can be explained by small variations due to host-specific adaptations and geographical isolation likely contributing to its divergence from the main cluster. Additionally, differences in host specificity (e.g., *B. taylorii* infecting rodents versus *B. quintana* infecting humans) and geographical separation likely drove genetic divergence in genes such as *gltA*, *nlpD*, and *bqtR*, explaining the distinct clustering of these strains[52,54,55]. In our study, the *nlpD* gene had 99.8% similarity (highest among all) with the *gltA* gene, and the *bqtR* gene showed a similarity of 99.7%. It has been shown that *rpoB* and *gltA* were the most effective markers for distinguishing between the 17 species and subspecies of the genus *Bartonella*[23]. Researchers identified a new *Bartonella* species (*Candidatus B. hemsundetiensis*) in *Myotis daubentonii* bats from Finland by analyzing *rpoB* and *gltA* sequences. Thus, multi-locus sequencing provides a valuable balance by potentially offering more reliable assessments of *Bartonella* diversity compared with single-locus methods[22]. This feature is highly advantageous in epidemiological studies, as it enables the tracking of transmission patterns and the examination of relationships between hosts.

Echocardiography is essential in diagnosing BCNE. Generally, it confirms the presence of endocarditis, assesses endocardial involvement, and guides treatment decisions[56,57]. Transthoracic echocardiography (TTE) however, has a limitation in detecting small vegetations, and hallmark lesions of IE, identifying only 25% and 70% of vegetations less than 5 mm, and between 6 and 10 mm[57]. In contrast, TTE detects 90–96% of the vegetations on native valves regardless of their size[58]. The size of vegetation in IE can vary depending on the stage of the disease. They may not be visible or very small in the early phases[59]. A previous study reported that 93.7% of BCNE cases with vegetation of 17.6 ± 11.3 mm were detected using echocardiography[60]. Our patient presented two large (>10 mm) lesions simultaneously. A similar rare case was described previously where a patient with infective endocarditis developed vegetative lesions on the pacemaker electrode[61]. In the present case, the patient had prior antibiotic use, but because he was referred to different physicians it was not possible to track his medication history. Monitoring vegetation size with TTE can be valuable for assessing the effectiveness of antibiotic therapy and predicting the likelihood of complications. This information can assist in making treatment choices and enhancing patient outcomes[62]. Larger vegetations, also increase the chances of other complications such as heart failure, renal failure, and neurological issues[62–64].

Similar to the present patient, there are reported cases of endocarditis due to *B. quintana* for which no epidemiologic risks (such as alcoholism or homelessness) were identified[65,66].

It is known that culturing *Bartonella* from valve samples is challenging due to the bacterium's fastidious nature and slow growth, especially when tissue samples are heavily degraded by prior antibiotic use[67]. Given these challenges and the high sensitivity of probe-based real-time PCR, we opted for molecular detection. The reason behind the failure in the isolation of bacteria in the microbial culture could be that usually when there is endocarditis and vegetations, bacteremia is already gone, as evidenced by IgM seronegativity. This has been observed both in humans and dogs.

Due to the timing of the sampling process coinciding with the outbreak of COVID-19 in Iran and the subsequent strain on the country's healthcare system in managing the pandemic, we were unable to expand our sample size and obtain a more comprehensive understanding of the prevalence of *Bartonella* endocarditis in Iran. In addition, because of limited resources, we could not test the samples for other potential causative agents such as *Coxiella burnetii* and *Chlamydia* pathogens to the cause of BCNE in the rest of the patients.

Conclusion

We present for the first time the involvement of *Bartonella* in BCNE in Iran. Future research on at-risk populations, as well as domestic and wild mammals as potential reservoirs, is recommended.

Data availability

All data generated or analyzed during this study are available within the main text of the article.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The Ethics Review Board of the Hamadan University of Medical Sciences, Hamadan, Iran approved the present study (Ethical approval codes: IR.UMSHA.REC.1398.1021). Ethical Review Board approved informed consent taken from all the participants and all experiments were performed following relevant guidelines and regulations.

Declaration of generative AI and AI-assisted technologies in the writing process

Generative AI and AI-assisted technologies were not used in the writing process.

Additional information

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