







Fastidious Bacterial Pathogens in Replaced Heart Valves: The First Report of *Bartonella quintana* and *Legionella steeli* in Blood Culture-Negative Endocarditis from Iran

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Abstract

Background: Infectious endocarditis is a rare infectious disease of the cardiac endothelium, but blood culture-negative endocarditis (BCNE) should be considered.

Objectives: This study aims to determine the prevalence of possible infectious agents of BCNE.

Methods: Real-time PCR and PCR sequencing were used to identify *Coxiella burnetii*, *Tropheryma whipplei*, *Brucella* spp., *Legionella* spp., and *Bartonella* spp. 16srRNA sequencing was used for the samples with negative results of PCR sequencing and real-time PCR.

Results: Among the 59 positive results, the rates of *Bartonella* spp., *Legionella* spp., and *Brucella* spp. were 8.5%, 5.1%, and 3.4%, respectively. *C. burnetii* and *T. whipplei* were not detected. 16srRNA sequencing revealed 1 *Stenotrophomonas maltophilia*, 1 *Aeribacillus* spp. and 1 uncultured bacterium.

Conclusions: The rate of some bacteria that are difficult to detect with routine laboratory tests was high in our study, and it seems that hospitals should use dedicated technology to detect BCNE pathogens.

Keywords: Endocarditis, BCNE, Bacterial Pathogen, *Bartonella quintana*, *Legionella steeli*

1. Background

Endocarditis is a rare infectious disease of the cardiac endothelium. The incidence rate ranges from 1.5 cases to 9.6 cases per 100,000 people, but the mortality rate is nearly high (about 25%) (1). Usually, a new cardiac problem with symptoms of high fever, sepsis, and systemic problems represents the likelihood of infective endocarditis (2). Symptoms are often ambiguous, so recognition is sometimes delayed, which can affect the quality of life (3). Early detection of infective endocarditis is critical, as treatment should be administered at a specific time to achieve the correct outcome (1). Different laboratory techniques such as culturing,

immunohistochemistry and immunofluorescence tests, serology, and molecular techniques are used for the diagnosis of endocarditis. Among the identification methods, isolation of bacterial strains by the culture of cardiac vegetation is a relatively available method to detect infective endocarditis. However, the negative result of culture does not rule out endocarditis (4). In other words, blood culture-negative endocarditis (BCNE) should be considered as a possible cause and contestable matter. Antibiotic use, bacterial growth conditions, and immunocompromised status are some of the BCNE etiologies. Mitral and aortic valves are most commonly affected, and tricuspid endocarditis has been reported in a few studies (5). Different microorganisms which

cause BCNE include *Coxiella burnetii*, *Bartonella* species, *Mycobacteria*, and *Tropheryma whippelii* (6).

Using only culture-dependent methods, as is common in most diagnostic centers, could be unreliable. Therefore, to obtain reliable results, molecular methods, including real-time PCR, should be performed. In this way, fewer false-negative results are obtained (7). The mortality rate of 20% in endocarditis and the severity of the disease may be because treatment could depend heavily on the detection of the microorganisms. Therefore, the use of an appropriate detection method is critical (8). Recent studies of patients with overt endocarditis symptoms who have a negative cultural and serologic test and a positive 16S ribosomal ribonucleic acid (16SrRNA) result indicate that molecular techniques are the best method for detecting BCNE (9). The state of immunosuppression could affect the serological result and negative results may mislead the physician to recognize the disease and apply the appropriate treatment (10). In addition to increasing biosafety and reducing the subculture of microorganisms, the use of PCR testing could increase the rate of confirmed positive BCNE tests up to 24.3% (11). There are a few studies about the rate of BCNE in Iran, which reported high rates, about 35% (12). In recent years, most investigations of endocarditis in Iran have used methods other than molecular testing to detect endocarditis. In 2010, the use of serological and microbiological tests showed that *Brucella* spp. was isolated in 2% of endocarditis cases (13). Another study in 2017 also showed a high prevalence of blood-negative culture endocarditis based on microbiological tests (14). However, in 2018, molecular tests showed the presence of some difficult-to-culture-bacteria including, *C. burnetii* (30.77%) (15) and as mentioned above, the importance of using molecular methods should be considered a definite detection method.

2. Objectives

This study aims to assess the prevalence of possible infectious agents causing endocarditis in patients who had heart surgery and negative culture history.

3. Methods

3.1. Patients and Histopathology

Samples selected in this investigation referred from 2012 up to 2018 to Rajaie Cardiovascular Medical and Research Center as one of the referral centers in Iran which were reserved in paraffin-embedded tissue collection of this center. Including criteria for selection was the

presence of inflammation in pathologic investigations based on the modified Duke criteria and negative results in blood culture tests. All parts of the sample selection in this study were approved by the Ethics Committee of the Pasteur Institute of Iran (Code: IR.PII.REC.1399.051).

3.2. DNA Extraction from Paraffin-Embedded Tissue Samples

To extract DNA from paraffin-embedded samples, tissue cores or microdissected tissue was cut into small parts and then subjected to xylene treatment, which dissolves the paraffin from the tissue, and then rehydrated using a series of ethanol washes (16). Genomic DNA was extracted from proceed samples using QIAGEN DNeasy® Blood & Tissue (Qiagen, Germany), according to the manufacturer's instructions. All extracted DNA was stored at -20 °C until molecular testing.

3.3. Molecular Identification of Bacterial Pathogens

Different molecular techniques were used to identify bacterial agents. Besides the real-time PCR method, for detecting *Coxiella burnetii*, *Tropheryma whippelii*, and *Brucella* spp., PCR sequencing was performed to identify *Legionella* spp. and *Bartonella* spp. since detecting the exact species of the mentioned bacteria was important in the current study. Also, for the samples with negative results of PCR sequencing and real-time PCR, 16srRNA PCR was used to determine the bacterial causative agent. The list of primers is available in Table 1.

3.4. PCR Sequencing Assay for Detection of *Legionella* spp. and *Bartonella* spp.

PCR tests were performed to detect *Legionella* (23S-5S rRNA gene) and *Bartonella* spp. The optimized PCR reaction conditions were 95°C for 60 s, followed by 40 cycles of 95°C for 30 s, 50°C (*Legionella*) and 60°C (*Bartonella*) for 30 s, and 72°C for 30 s. The list of primers is available in Table 1. All the tests have been done by Labcyler Gradient SenSoQuest (Yechizi Co, Germany). Specific positive controls were used in all tests. Following gel electrophoresis, all positive samples were sequenced.

3.5. Real-time PCR Assay for Detection of *Coxiella burnetii*, *Tropheryma whippelii*, and *Brucella* spp.

The bacterial genera, including *C. burnetii*, *T. whippelii*, and *Brucella* were investigated through real-time PCR. Reactions were prepared using the following mixture: 12.5 µL of 2x Master Mix (RealQ Plus for Probe, Ampliqon, Denmark), 900 nM from each primer, 200 nM probe, and 5 µL of DNA templates. The Corbett 6000 Rotor-Gene system (Corbett, Victoria, Australia) was used for performing the tests, with a final volume of 25 µL. The PCR amplification program was 10 minutes at 95°C, followed by 45 cycles of 15 s at 94°C and 60 s at 60°C.

Table 1. Primer Sequences Used in This Study

Gene	Forward Primer	Reverse Primer	Probe
<i>IS1111 (Coxiella)</i>	AAAACGGATAAAAAGAGTCTGTGGT	CCACACAAGCGGATTTCAT	6-FAM-5'-AAAGCACTCATTGAGCGCCGCG-3'TAMRA
<i>IS711 (Brucella spp.)</i>	GCTTGAAGCTTGCGGACAGT	GGCTACCGCTGCGAAT	6-FAM-5'-AAGCCAACACCCGGCCATTATGGT-3'TAMRA
WISP family protein (<i>Tropheryma whipplei</i>)	TTGTGTATTGGTATTAGATGAAACAG	CCCTACAATATGAAACAGCCTTTG	6-FAM-5'-GGGATAGAGCAGGAGGTGTCTGTCTGG-3'BHQ-1
16S rRNA gene (Legionella)	AAGATTAGCCTGCGTCCGAT	GAGGGTTGATAGGTTAAGAGC	-
Bartonella 16srRNA	TTAGAGTGAGCGGCAAAC	TACCGTCATTATCTCACCG	-
16S rRNA	AGAGTTTGATCMTGGCTCAG	AAGGAGGTGWTCARCC	-

3.6. 16srRNA Sequencing

PCR test followed by sequencing was used to detect the 16srRNA gene to determine the causative bacterial agent. The optimized PCR reaction conditions were 95°C for 60 s, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. All the tests have been done by Labcycler Gradient SenSoQuest (Yechizi Co, Germany). Specific positive controls were used in all tests. All positive samples were sequenced. Sequenced on both strands using the BigDye Terminator v. 1.1 Cycle Sequencing Kit® (Applied Biosystems) and an ABI PRISM 3130xl Sequencer® (Applied Biosystems) were sequenced. The sequences thus obtained were compared with the sequences available in GenBank using the BLASTN program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Species identification was based on 99% 16S rDNA sequence identity with the GenBank prototype sequence, and genus identification was based on 97%.

4. Results

4.1. Patients and Sampling

Over the study period, eight thousand patient medical reports between 2012 to 2018 were checked out, and 190 samples were selected from patients who had valve replacement surgery with histopathological evidence of inflammation and according to the modified Duke criteria. Out of 190 samples, 59 specimens had a culture-negative result and were without any evidence of confirmatory data on a causative agent. 18 (26.4%), 21 (30.8%), 4 (5.8%), 5 (7.3%) were categorized into sub-acute, chronic inflammation, acute inflammation, and acute-chronic inflammation, respectively. The rest belonged to other pathological reports without considering the inflammation or bacterial presence (Figure 1).

4.2. Molecular Detection of Bacterial Agents

4.2.1. Results of PCR Sequencing Assay

The PCR sequencing results indicated that among 59 samples with negative culture results, *Bartonella* and

Legionella were detected in 5 (8.5%) and 3 (5.1%) samples, respectively. According to sequencing results, all samples of *Legionella* were *L. steelei* and all the *Bartonella* samples were *Bartonella quintana*.

4.2.2. Results of Real-time PCR Assay

According to the results of the real-time PCR assay, *C. burnetii* and *T. whipplei* were not detected while *Brucella* was detected in 2 (3.4%) samples.

4.2.3. Results of 16srRNA Sequencing

16srRNA sequencing assay revealed that there were seven samples with positive results and the sequencing showed one *Stenotrophomonas maltophilia*, one *Aeribacillus* spp., and one uncultured bacterium.

5. Discussion

BCNE could be considered as one of the contestable diseases since the diagnosis is usually associated with some difficulties. The use of antibiotics and the presence of fastidious bacteria and intracellular microorganisms that cannot be detected in blood culture tests are some of the reasons that complicate the diagnosis. The rate of BCNE is reported to be about 35% (7), however, this rate varies from 10.2% to 75.6% in different countries because of methods and strategies for detection, rates of fastidious microorganisms, history of antibiotic use specifically before blood collection, methods of sampling, and the presence of noninfectious or unknown pathogens may vary in different regions (17). In addition, the rate of IE is considerable in southern Mediterranean countries, and most patients with IE were younger than 40 years of age (18). The College Center of Aix, one of the valid BCNE detection centers in France, reported 1500 endocarditis with negative culture results from 2010 to 2015 (12). The use of different identification methods in this center could increase the probability of diagnosis. In other words, they indicate that, in addition to serological methods, PCR is a

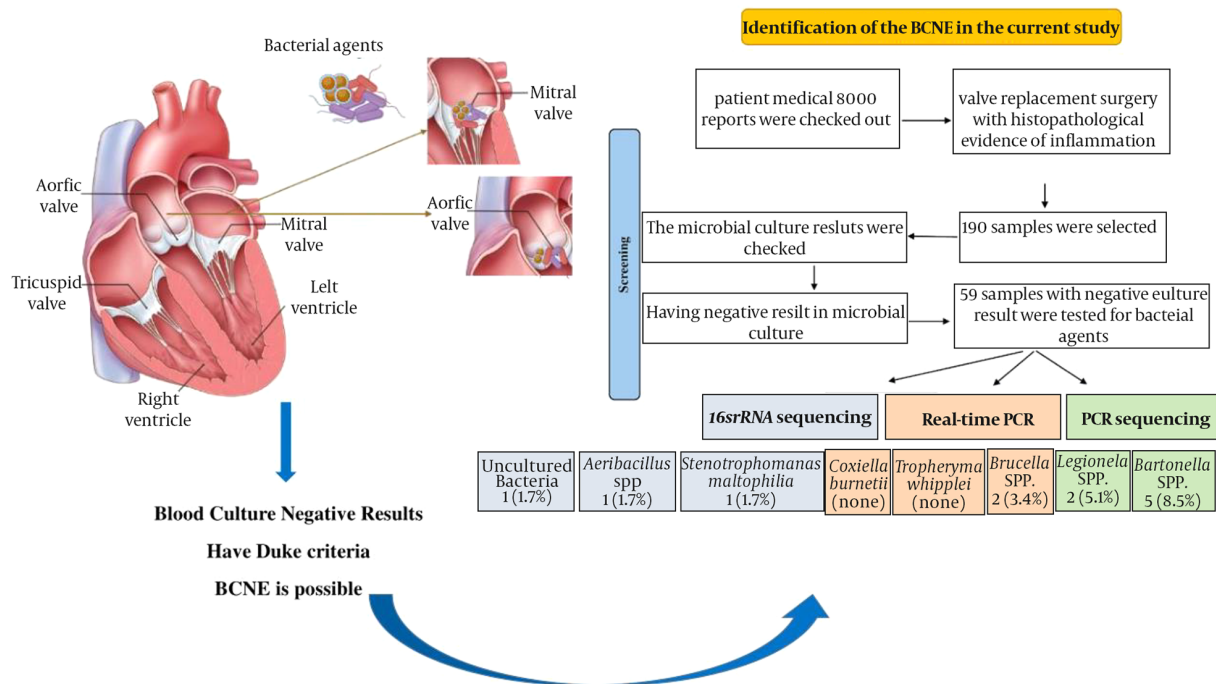


Figure 1. The procedure of BCNE detection and the rates of bacterial agents were identified in the current study

reliable method for detecting challenging microbial BCNE pathogens.

In the current study, 8000 patient medical reports were reviewed, and 190 samples were collected. Of the 190 samples, 59 samples tested negative for bacterial pathogens with culture results. *Bartonella*, *Legionella*, and *Brucella* were detected in 5 (8.5%), 3 (5.1%), and 2 (3.4%) specimens, respectively. The rates of *Bartonella* and *Legionella* are remarkable in the current study. Similar results are shown in other studies. Pecoraro et al. reported *Bartonella* as the most common cause of endocarditis (19). On the other hand, the results showed that *16srRNA* sequencing is not a helpful method to detect the different causative agents of BCNE, since it was only detected in 7 samples. Among these seven samples, one *Stenotrophomonas maltophilia*, and one *Aeribacillus* isolate were detected.

In the current study, the rate of *Bartonella* was 8.5%. *Bartonella* spp. was first discovered as endocarditis causative agent in 1993 (20). Up to 2006, 120 *Bartonella* spp. were detected as BCNE causative agents (21). Notably, *Bartonella* is the cause of nearly 12% to 60% of BCNE cases, making it the second most common cause of BCNE after *Coxiella burnetii* (22). *Bartonella quintana* and *B. henselae* were the most prevalent agents (23). In the current study, we used two different methods (using species-specific

primers and sequencing) to increase specificity. Using an appropriate method to detect bacteria is critical to reduce the number of false positives and negatives. For example, serologic tests are useful laboratory methods for detecting *Bartonella* in endocarditis. However, the incidence of cross-reactivity between *Bartonella* and some other bacteria such as *Chlamydia* and *C. burnetii* is problematic. Based on various studies, the tests from RT-PCR are useful, sensitive, specific, and rapid methods for the detection of *Bartonella* in endocarditis. Real Time-PCR is a specific test for the detection of *Bartonella* in heart valve samples (92%) (24); while for the blood or serum samples, RT-PCR is less specific (33% and 36%, respectively) (25). Compared with *16srRNA*, real-time PCR is a more sensitive test; however, all positive real-time PCR tests also had positive results for *16srRNA* sequencing in the current study. On the other hand, endocarditis caused by *Bartonella* occurs more frequently in patients with valvular insufficiency and valvular heart surgery, which is why detection of *Bartonella* at the right time would be so important. In the future, the development of PCR methods to detect *Bartonella* could be a rapid, reliable, and preventive test to detect endocarditis in our hospitals.

In the present study, *Legionella* was detected in 5.1% of the samples with negative culture results. Endocarditis caused by *Legionella* has rarely been reported. It was first

discovered in 1984, and only 16 cases had been reported by 2016. Of these 16 patients, only two had healthy and normal heart valves, and the others had a history of valve surgery (26). PCR and culture-based methods are the gold standards for *Legionella* detection. In addition to these gold-standard methods, the detection of bacterial antigens in urine samples is also a routine method (27), however, the delay in the increase of antibodies remains a significant problem. In other words, a positive result on pathologic testing, observation of bacteria through an electronic microscope, and positive PCR results are the accurate ways to detect *Legionella* in endocarditis (28). As far as we know, this is the first report of the detection of *Legionella* species other than *L. pneumophila* causing BCNE in Iran.

In this study, *Brucella* was detected in 3.4% of BCNE cases. Endocarditis caused by *Brucella* is endemic in some regions such as Egypt, and it is usually a common causative agent of BCNE, after *Bartonella* (8). Although *Brucella* was less common, *Brucella melitensis* has been reported to be associated with a history of valvular heart surgery, which can cause more severe disease. In other words, without effective and timely antimicrobial treatment and valve surgery, the mortality rate could be as high as 80% (29). Iran is an endemic region for brucellosis, and the Ministry of Health and Medical Education has classified the country into four areas: very high, high, average, and low prevalence. For this reason, it seems that endocarditis may have a high prevalence in our country. Misdiagnosis is common because brucellosis has no pathognomonic symptoms. Therefore, the use of a rapid and sensitive method is crucial.

We also found *Stenotrophomonas maltophilia* among the BCNE samples. *Stenotrophomonas* forms a biofilm that can adhere to various surfaces and has a high resistance to antimicrobial agents. *S. maltophilia* has a low probability of causing endocarditis as a nosocomial pathogen. Although it could be considered a multidrug-resistant isolate, *Stenotrophomonas* is an opportunistic bacterium and should be considered in patients with underlying diseases (30). In other words, intrinsic resistance leads to an increase in morbidity and mortality among BCNE cases; therefore, timely identification of this organism is necessary to improve medical management. It seems that the development of real-time PCR-based methods may have advantages in reducing mortality and morbidity rates in the future.

5.1. Conclusions

According to the current results, the factors causing endocarditis could be different. The rate of *Legionella* and *Bartonella quintana*, which are difficult to detect by routine

laboratory tests, was high in our study, and to the best of our knowledge, this is the first report of *Legionella steeli* and *Bartonella quintana* among BCNE cases in Iran. It seems that hospitals should use special technology to detect these BCNE pathogens. In this case, surgery and valve replacement could be avoided. Systematic examination of samples of replaced heart valves to detect fastidious bacteria in cardiovascular centers, consideration of the bacterial group HACEK, and timely detection of BCNE pathogens could prevent heart valve surgery and improve the success of antimicrobial treatment.

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Footnotes

Authors' Contribution: Performed the experiments: MR, SE, MSSN, and HRP. Sampling: NA, PM, KM, NM, and MM. Data analysis: ShA, SE, and MR. Writing of the manuscript: ShA. Revised manuscript: MR, and EM. Conceived and designed the experiments: MR, SE, and EM.

Conflict of Interests: The authors declare no conflict of interest (financial and non-financial).

Data Reproducibility: All data generated or analyzed during this study are included in this published article. Also, the raw data of the current study are not publicly available due to their proprietary nature, privacy, and ethical concerns, but are available from the corresponding author on reasonable request.

Ethical Approval: The experimental protocols were established following the Declaration of Helsinki and approved by the Ethics Committee of Pasteur Institute of Iran (IR.PIL.REC.1399.051) and all methods were performed following the relevant guidelines and regulations.

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