Tick-borne pathogens *Bartonella* spp., *Borrelia burgdorferi* sensu lato, *Coxiella burnetii* and *Rickettsia* spp. may trigger endocarditis

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Conflict of interest
None declared

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Abstract

Background. Infections caused by tick-borne pathogens such as *Bartonella* spp., *Borrelia burgdorferi* s.l., *Coxiella burnetii*, and *Rickettsia* spp. are capable of causing serious lesions of the mitral and aortic valves, leading to a need for valve replacement.

Objectives. The aim of the study was to determine whether such cases are sporadic or frequent. An additional goal was to establish effective diagnostic methods to detect these infections.

Material and methods. The study involved 148 patients undergoing valve replacement. Blood samples were drawn for serological testing. Samples of the removed mitral and aortic valves were tested with polymerase chain reaction and immunohistochemical staining.

Results. Specific antibodies to *Bartonella* spp. were detected in 47 patients (31.7%) and in 1 of the healthy controls (1%) ($p < 0.05$). Antibodies to *B. burgdorferi* spirochetes were found in 18 of the patients (12.2%) and in 6 blood donors from the control group (5.8%) ($p < 0.1$). Antibodies to *Rickettsia* spp. were detected in 12 (8.1%) and to *C. burnetii* phase I and II antigens in the serum of 1 patient. All the participants in the control group were seronegative to *C. burnetii* and *Rickettsia* spp. antigens. Polymerase chain reaction (PCR) tests for detection of *Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii* and *Rickettsia* spp. DNA in the valve samples were all negative. Inflammation foci with mononuclear lymphoid cells in the aortic and mitral valves were seen in sections stained with hematoxiline and eozine. In sections dyed using the indirect immunofluorescence method with hyperimmune sera, *Bartonella* spp. and *Rickettsia* spp. were found.

Conclusions. The results obtained indicate that laboratory diagnostics for patients with heart disorders should be expanded to include tests detecting tick-borne zoonoses such as bartonelloses, Lyme borreliosis, rickettsioses and Q fever.

Key words: endocarditis, tick-borne diseases, heart valve

There is increasing evidence that infectious diseases transmissible from animals to humans may trigger various heart disorders. Very often fastidious, nonculturable pathogens, such as *Borrelia burgdorferi* sensu lato, *Coxiella burnetii*, *Bartonella* spp. and *Rickettsia* spp. are responsible.[1,2] This is the reason infections with these bacteria are very frequently undiagnosed. The symptoms of endocarditis and valvular heart disease may indicate the bacterial etiology of the disease, and detection of significant titers of specific antibodies allows the origin of the disease to be identified.[2–4]

The aim of the present study was to establish whether tick-borne infections can contribute to serious heart disorders, resulting in a need for heart valve transplantation, and to determine whether diagnoses of *Bartonella* spp., *B. burgdorferi*, *C. burnetii* or *Rickettsia* spp. infections should be considered in every case of infectious endocarditis with negative blood cultures.

Material and methods

The patients

A group consisting of 148 patients undergoing valve replacement due to clinically recognized infective endocarditis were treated in the Cardiac Surgery and Transplantology, Department of the National Institute of Cardiology in Warszawa, Poland. The age range of the patients was 25–86 years, with the great majority from 66 to 86 years old. The group included 58 inhabitants of small villages (39.2%) and 90 residents of big cities (60.8%). The patients qualified for surgery did not have any records of previous antibiotic treatment.

Sera from 101 blood donors (47 women and 54 men) aged 31–65 years old (median age 48.5 years) were included as a control group in the serologic part of the study. The controls in the molecular and histological part of the study were samples of the mitral valves, aortal valves and myocardia from 70 individuals qualified as organ donors for transplantation after their deaths in accidents. These tissues were included after undergoing a number of serological, molecular, and bacteriological examinations. Infections with aerobic and anaerobic bacteria, viral or fungal infections were excluded by cultivating the blood and tissues.

The study was approved by the Institutional Review Board (IRB) at the National Institute of Public Heath – National Institute of Hygiene, Warszawa, Poland (approval No. 1/2012, issued on June 28, 2012).

Material

Blood samples were drawn for serological testing. Samples of the removed mitral and aortic valves were collected. Tissue samples were frozen at –75°C until tested.

Histology

Double imprint preparations were prepared from all the tissue samples. The slides were air-dried. Half of the slides were stained with hematoxylin and eosine and the other half were stained using the indirect immunofluorescence method.

Hematoxylin and eosin staining

The slides were fixed with acetone, washed with water for 5 min, then stained with Meyer's hematoxylin and 1% eosin and washed again with water. Next, the slides were dehydrated in ethanol (96–100%), cleared with xylene and embedded in Canadian balsam.

Immunohistochemical staining

The slides were fixed in a 1:1 mixture of methanol and acetone for 1 min at room temperature. Next, the slides were covered with human hyperimmune sera against *Bartonella* spp. (diluted 1:1600 in PBS), *B. burgdorferi* (1:400 dilution), *C. burnetii* (1:1024) or *Rickettsia* spp. (1:80) and incubated in a wet chamber for 20 min at 37°C.

The slides were washed 3 times in PBS with gentle agitation and then stained with diluted (1:80) goat anti-human fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich, St. Louis, USA) and incubated in a wet chamber for 20 min at 37°C. The slides were washed 3 times in PBS with gentle agitation and embedded in glycerol, then immediately examined under a fluorescence microscope (Nikon, Tokyo, Japan) at 40×10 magnification.

Polymerase chain reactions

Each tissue sample was cut and 30 mg was homogenized. DNA was extracted with a QIAamp Tissue kit (QIAGEN Gmbh, Hilden, Germany) according to the manufacturer's instructions. To ascertain whether the size of the valve samples was sufficient, DNA was extracted and amplified from 300 mg of 5 tissue samples.

The following primers were used to detect DNA of:

- *Bartonella* spp.: BhCS.781 and BhCS1137n primers for citrate synthase gene fragments;
- *Borrelia burgdorferi* sensu lato: L2 and P1 primers for 16 S r RNA and OA149 and OA319 specific primers for the *OspA* gene fragments characteristic of all *Borrelia burgdorferi* s. l. genospecies;
- *Coxiella burnetii*: isIIIIf and isIIIIr for the *htpAB* gene fragment (3,5,6,7,8) (Table 1).

The 50 µL reaction mixtures contained 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% gelatin, 200 µM dNTPs, 50 pmol of each primer and 1.5 U Taq DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, USA). An aliquot of 5 µL of extracted DNA template was added to each reaction mixture. Each polymerase chain reaction (PCR)

Table 1. Primers and nucleotide sequences used in the study

Pathogen	Primers	Gene fragment (size bp)	Nucleotide sequences	References
Bartonella spp.	Bh.CS.781p BhCS.1137n	Citrate synthase gene (400)	5′-CCTATGGCTATTATGCTTGC 5′-AATGCAAAAGAACAGTAAACA	5
Borrelia burgdorferi sensu lato	L2 P1	16S rRNA (600)	5′-GGTCAAGACTGACGCTGAGT 5′-TCGCTTTGTACAGGCCATTG	6
	OA149 OA319	OspA gene	5′-TTATGAAAAAATATTTATTGGGAAT 5′-CTTTAAGCTAAGCTTGTCTACTGT	7
Coxiella burnetii	isIIIIf isIIIIr	htpAB	5′-CGCTCTCGTTTATGCGAGC 5′-CCAACAACACCTCCTTATTC	3
Rickettsia spp.	RpCS. 409D RpCS. 1258n	Citrate synthase gene (850)	5′-CCTATGGCTATTATGCTTGC 5′ATTGCAAAAAGTACAGTGAACA	8

test included negative (water) and positive controls containing the DNA of *B. afzelii*, *B. garini*, *B. henselae*, *C. burnetii* Henzerling strain and *Rickettsia conori* H24 strain, all from the collection of the National Institute of Public Heath – National Institute of Hygiene. For *B. burgdorferi* and *Rickettsia* spp. the cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 1 min denaturation at 95°C, annealing for 1 min at 55°C, elongation for 1 min at 72°C and final elongation for 7 min at 95°C. For *Bartonella* spp. the cycling conditions were as follows: 10 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C and finally 10 min at 72°C. For *C. burnetii* the cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 57°C, 1 min at 72°C, and finally 7 min at 95°C. Polymerase chain reactions were performed in a Mastercycler EP Gradient PCR/thermal cycler (Eppendorf AG, Hamburg, Germany).

Serology

We looked for serum antibodies to *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Bartonella* spp. and *Coxiella burnetii* in the patients' sera. To detect IgG *Rickettsia* spp. antibodies, micro immunofluorescence (MIF) – the reference method for a serological diagnosis of rickettsioses – was used. Inactivated *R. typhi* and *R. rickettsii* antigens were used (Rickettsia IFA IgG, Focus Diagnostics, Diasorin Molecular LLC, Cypress, USA). Control sera were delivered by the manufacturer. The screening was performed at a titer of 1:16. All serum samples negative in this titer were regarded as negative to both groups of rickettsiae. According to Unité des Rickettsies (Marseilles, France) an IgG titer ≥64 is considered indicative of infection by these specific *Rickettsia* species.[3]

Levels of *Bartonella* spp. IgG antibodies were determined by indirect immunofluorescence assay (Bartonella IFA IgG; Focus Diagnostics). Vero cells infected with *B. henselae* or *B. quintana* strains were used as the antigens. A titer of IgG antibodies ≥64 was considered positive. Positive and negative controls were delivered by the manufacturer.

B. burgdorferi IgM and IgG antibodies were tested with ELISA kits (EIA Borrelia 14kDa+OspC IgM and EIA Borrelia IgG+VisE, DRG-Medtek, Marburg, Germany). Positive results were confirmed by the western blot method (Euroline Borrelia-RN-AT IgM, Euroline Borrelia-RN-AT IgG; EUROIMMUN, Lübeck, Germany). Results were interpreted as seropositive according to the criteria of the German Society of Hygiene and Microbiology.[9]

The statistical analysis (χ^2 test) of the serologic test results was done using Statistica software (StatSoft Inc., Tulsa, USA). P-values <0.05 were considered statistically significant.

Results

The patients

The blood cultures of all 148 patients admitted to the clinic for valve replacement with symptoms of infective endocarditis were negative. During and after surgery; however, all the patients received 1 g of cefazolinum as perioperative antibiotic therapy, sometimes up to 1–2 days, to prevent the infection from spreading. Five patients were additionally treated with gentamicin/amoxycilline + clavulonic acid/ceftriaxone due to high temperatures (Table 2).

Serology

All the patients were examined serologically for the presence of specific antibodies to *Bartonella* spp., *B. burgdorferi*, *C. burnetii* and *Rickettsia* spp. Among the inhabitants of big cities, 42 (67%) were found to be seropositive for these tick-borne pathogens, as were 21 of the residents of villages (33%). Specific antibodies were found in the sera of 63 patients (42.6%). The seropositive group consisted of 47 patients (31.7%) with specific *Bartonella* spp. antibodies, 18 patients (12.2%) with *B. burgdorferi* s.l. antibodies, 12 patients (8.1%) with antibodies to *Rickettsia* spp., and 1 patient (0.7%) with specific *Coxiella burnetti* antibodies. The following ranges of the serum antibody titers were detected: *Bartonella* spp. 64 – 2048, *Rickettsia* spp. 64 – 128,

Table 2. Patients treated with antibiotics before surgery

Patient's number/initial	Additional antibiotics[a]	Duration	Presence of antibodies to:
31/P.B.	amoxicillin+ clavulanic acid (Taromentin)	7 days	negative
45/J.S.J.	cefazolin (Tarfazolin, Biofazolin)	4 days	negative
58/N.W.	ceftriaxone (Biotraxone) cefazolin (Biofazolin)	1 day 1 day	*B. burgdorferi*
P77/O.S.	ceftriaxone (Biotraxone) cefazolin (Biofazolin)	7 days 1 day	*Bartonella* spp.
P78/Pa.	gentamicin cefazolin (Biofazolin)	1 day 1 day	*B. burgdorferi Bartonella* spp.

[a]All the patients were given 1-day treatment with 1 g cefazolinum before or during surgery.

Table 3. Patients with recognized Lyme borreliosis (positive results of a 2-step serological test for *Borrelia burgdorferi*)

Ordinal No.	Patient's number/ initials	Detected fractions[a]
1	P2	p17, p19, p25, p30, p31, p41, VlsE
2	P.kr	p100, p39, p18, VlsE
3	K.kr	p100, p25, VlsE
4	H.Gen.	p18, VlsE
5	P.B.	p18, VlsE
6	P.A	p100, p58. p39, p18, VlsE
7	M.C.	p100, p58. p39, p18, VlsE
8	K.A.	p100, p58, p41, VlsE
9	P58	p58, VlsE
10	P59	p58, p18, VlsE
11	P61	p100, p58, p39, p18, VlsE
12	P65	p100, p18, VlsE
13	P69	p100, p41, p18, VlsE
14	P78	p58, p18, VlsE
15	P109	p100, p25, p18, VlsE
16	P110	p100, p18, VlsE
17	P117	p18, VlsE
18	P122	p58, p39, VlsE
19	P126	p18, VlsE
20	P132	p18, VlsE
21	P141	p18, VlsE

[a] specificity confirmed by immunoblotting: The presence of 2 fractions from the following: p83/100, p58, p43, p41 int., p39, OspC, DbpA (p17/18), VlsE indicates an IgG positive result.

C. burnetii phase I 32 and phase II 256. The specificity for *B. burgdorferi* was confirmed by immunoblotting (Table 3). The presence of antibodies to *B. burgdorferi* p18 and VlsE antigens is characteristic of chronic infections. According to the Modified Duke Criteria for the Diagnosis of Infective Endocarditis, 63 patients (42.6%) fulfill the criteria for infective endocarditis: A characteristic echocardiogram picture (the major clinical criterion) and the presence of significant levels of specific antibodies to *Bartonella* spp., *B. burgdorferi* s.l., *Rickettsia* spp. or *C. burnetii* (a minor criterion).[10,11]

In the study group, co-infections of *Bartonella* spp. with *B. burgdorferi* s.l. were detected in 8 patients; with *Rickettsia* spp. in 3 patients; and with *C. burnetii* in 1 patient. Also, 1 patient presented specific antibodies to the antigens of *Bartonella* spp., *B. burgdorferi* s.l. and *Rickettsia* spp., indicating co-infection with 3 pathogens.

In the control group (the blood donors), specific IgG antibodies to *B. henselae* antigen were detected in a titer of 128 in 1 person (1%; p-value <0.05). Specific IgG antibodies to *B. burgdorferi* sl. were detected in the sera from 6 blood donors (5.8%; p-value <0.1). All the control sera were seronegative to *C. burnetii* and *Rickettsia* spp. antigens.

Histopathology

Macroscopic evaluation of the valve samples obtained from the patients showed vegetation and other degenerative changes, mostly fibrosis and calcification. Light microscopic examination of hematoxylin & eosin stained valve preparations showed diffusely infiltrating mononuclear inflammatory cells. The infiltrates were composed predominately of mononuclear cells, including lymphocytes and plasma cells (Fig. 1A). In some patients mononuclear lymphoid cells were sparsely accompanied by polymorphonuclear leukocytes. Occasionally, small aggregates of lymphocytes and macrophages were found. Additionally, we observed numerous fibrocytes forming a cellular net with mononuclear inflammatory cells (Fig. 1B).

Immunofluorescent staining with specific *Bartonella* spp., *B. burgdorferi*, *Rickettsia* spp. and *C. burnetii* immune sera showed positive reactions to *Bartonella* spp. and *Rickettsia* spp. cells (Fig. 2A,B).

Structural valvular changes were not observed in the tissues from organ donors group.

Apparent discrepancies between serology and immunohistochemistry

Among the 63 patients seropositive to *Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii* or *Rickettsia* spp., the presence of bacterial cells was also found in some of the tested tissue sections. *Bartonella* spp. bacterial cells were detected in 9 valve sections removed from 47 seropositive patients; *Rickettsia* spp. bacteria were seen in 5 tissue samples

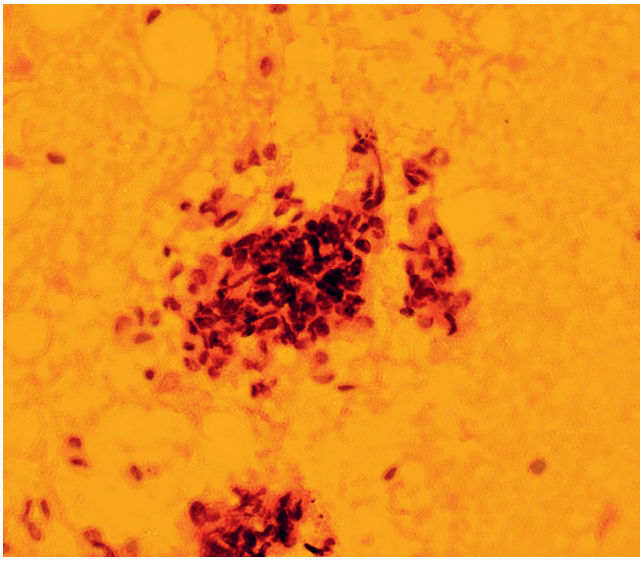

Fig. 1A. Sections of valves stained with hematoxylin & eosin (H&E) (×400): A. accumulation of mononuclear lymphoid cells in valve's section from patient seropositive to *Bartonella* spp. (serum titer 1:128)

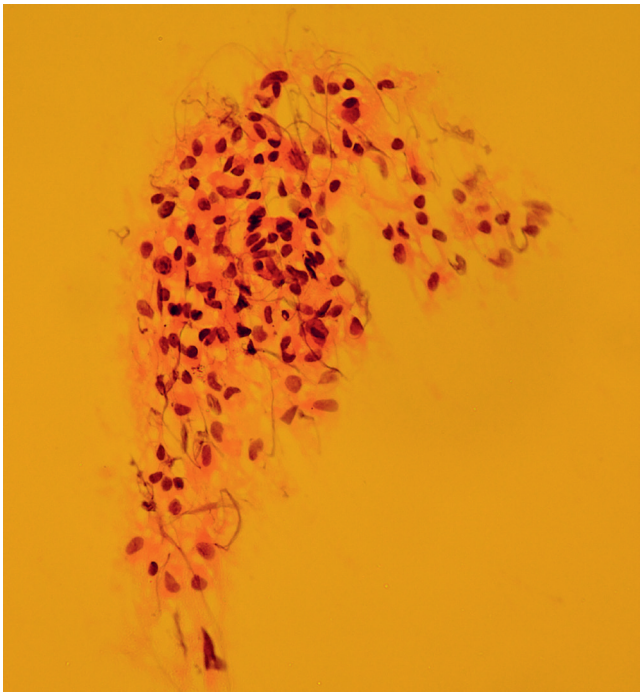

Fig. 1B. Sections of valves stained with hematoxylin & eosin (H&E) (×400): B. mononuclear inflamatory cells surrounded by fibrocytes in valve from patient seropositive to *Bartonella* spp. (serum titer 1:128)

from 12 seropositive patients; and *B. burgdorferi* spirochetes were observed in 1 out of 18 seropositive patients. *C. burnetii* bacteria were not found in any tissue section. These results indicate the different sensitivity of the tests employed rather than discrepancy.

Polymerase chain reactions

All tests performed to detect *Bartonella* spp./*B. burgdorferi* s.l./*C. burnetti*/*Rickettsia* spp. DNA were negative.

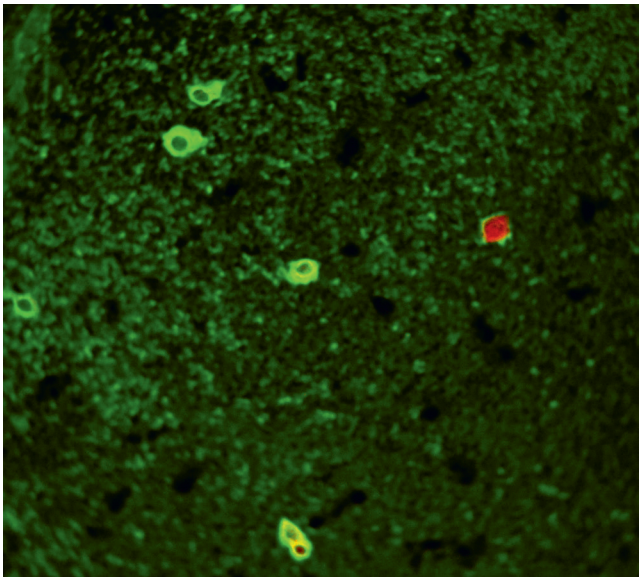

Fig. 2A. Sections of valve from patients with antibodies to *Bartonella* spp. (serum titer 1:128) and *Rickettsia* spp. antigents (titer 1:64), dyed with indirect immunofluorescence (×400): A. immunofluorescence of mononuclear cells with *Bartonella* spp. hyper immune serum

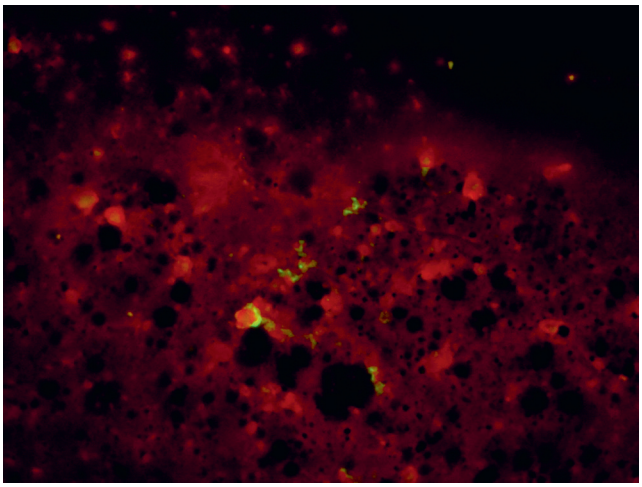

Fig. 2B. Sections of valve from patients with antibodies to *Bartonella* spp. (serum titer 1:128) and *Rickettsia* spp. antigents (titer 1:64), dyed with indirect immunofluorescence (×400): B. immunofluorescence with *Rickettsia* spp. hyper immune serum

Discussion

The aim of our study was to establish whether infections caused by tick-borne pathogens such as *Bartonella* spp., *B. burgdorferi* s.l., *C. burnetii* and *Rickettsia* spp. are capable of causing serious lesions of the mitral and aortic valves, leading to a need for valve replacement. One goal was to determine whether such cases are sporadic or frequent; an additional goal was to establish effective diagnostic methods to detect these infections.

The results obtained indicate that tick-transmitted pathogens (*B. burgdorferi* s.l., *Bartonella* spp., *Rickettsia* spp., *C. burnetii*) play a significant role in the development

of endocarditis, causing irreparable damage requiring valve replacement.

Out of 148 patients referred for valve replacement, 63 (42.6%) showed the presence of specific antibodies to *Bartonella* spp., *B. burgdorferi* spirochetes, *Rickettsia* spp. and *C. burnetii*. It is surprising that 67% of the patients infected with these tick-borne pathogens were residents of big cities, and only 33% live in the countryside. This can be explained as a consequence of lifestyle changes. Recently, society favors active recreation, various forms of tourism, extreme sports and exotic excursions. This sometimes has negative effects due to more frequent contact with new pathogens, creating new high-risk groups contracting tick-borne zoonoses. Wild animals (birds, mammals) can be reservoirs of the etiologic agents of these diseases. Many of these infections appear seasonally, during the active periods of various species of arachnids, which are very often the vector of various microorganisms. In our climatic zone ticks transmit infections from the spring to the autumn.

In Poland, Q fever endocarditis (caused by *Coxiella burnetii*) has never been diagnosed,[12] possibly because the *Coxiella burnetii* strains isolated in Poland represent phenotypes that correlate with acute disease; they are different from the strains that have appeared in France.[4,13] The "gold standard" for the diagnosis of an infection is the isolation of the etiologic agent in a culture. In the case of tick-borne diseases, such as rickettsioses, bartonelloses, Q fever and Lyme borreliosis, the causative microorganisms are rarely cultured. In addition, negative results may develop from sampling errors due to the bacteria being present in the tissues in low concentrations.[14–16]

The delay in the recognition of tick-borne diseases may be related in part to the fact that many cases appear to be asymptomatic or subclinical. When symptoms are present, the typical presentation of an undifferentiated febrile illness (including fever, headache, malaise, myalgias, and arthralgias) may be difficult to distinguish since these diseases have similar epidemiological distribution and are transmitted by the same ticks.[17–19] Moreover, infectious cardiac diseases may be para-infectious phenomena, e.g., inflammation, rather than the result of direct action by viable microorganisms.

Efforts to detect bacterial nucleic acids are also limited by many other factors. Some authors have reported that studies using single biopsies produce consistently lower estimates for intracellular pathogens than studies that examine multiple biopsies.[15,16,20,21] Molecular techniques such as PCR improve the likelihood of identifying an infectious agent, but should be used judiciously and in conjunction with traditional diagnostics such as culture, serology and histology. Knowing the limitations of PCR, it is important to take appropriate samples, selecting those that are most likely to have a significant bacterial DNA load.[18,22,23]

PCR testing is of relatively low sensitivity after antibiotic therapy. Patients with infective endocarditis due to *Bartonella* spp. are usually treated with aminoglycosides for more than 14 days or with doxycycline for 6 weeks in combination with gentamicin for 14 days.[19,24] Differences in the sensitivity of PCR in tissue specimens may be explained by the use of antibiotics prior to sampling, which may reduce the amount of bacterial DNA.

In addition, the results may vary depending on the type of tissue sample – e.g. vegetation or valve tissue. This implies that the type of tissue biopsied may be critical, with vegetation more likely than heart valves to yield a positive result. This may not be surprising, as vegetation is believed to contain dense clumps of bacteria (and hence high bacterial DNA loads) within a matrix of fibrin, platelets and erythrocyte debris.[15,25]

The principles of diagnosis and recognition of infective endocarditis caused by tick-borne pathogens have not been clearly established. In 2012, new criteria for recognizing Q fever endocarditis were proposed.[26] Currently, there is a need to establish criteria for *Bartonella* and *B. burgdorferi* endocarditis. Some suggestions concerning *Bartonella* were submitted by Raoult et al. in 2015; however, these need further analysis.[4,19,24]

Recently, it has been shown that many tick-borne zoonotic diseases contribute to valve and myocardium damage. It has been observed in the course of Lyme borreliosis, bartonelloses, rickettsioses and Q fever. These diseases are recognized as emerging or re-emerging infections and serious public health problems. If the role of tick-borne infections in the development of serious heart malfunctions is suspected, it should be obligatory to perform diagnostic serological tests, especially in cases of negative blood cultures. Generally, serological tests are fast and not as invasive as blood cultures. Performing a serological examination makes it possible to confirm cases of infective endocarditis and myocarditis and to apply antibiotic treatment before irreversible damage is done.

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