

Molecular Diagnosis of Infective Endocarditis by PCR Amplification and Direct Sequencing of DNA from Valve Tissue

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We used broad-range eubacterial PCR amplification followed by direct sequencing to identify microbial pathogens in heart valve material from 29 patients with histologically confirmed infective endocarditis and 23 patients free of infective endocarditis. Microorganisms cultured by conventional techniques matched those identified by PCR in 21 cases. PCR alone identified the causative agent in three cases (*Streptococcus bovis*, *Staphylococcus cohnii*, and *Coxiella burnetii*), allowing better patient management. PCR corrected the initial bacteriological diagnosis in three cases (*Streptococcus bovis*, *Streptococcus mutans*, and *Bartonella henselae*). Among the 29 cases of histologically confirmed infective endocarditis, PCR findings were positive in 27 cases and were consistent with the bacterial morphology seen at Gram staining (26 cases) or with the results obtained by immunohistologic analysis with an anti-*C. burnetii* monoclonal antibody (one case). In two other cases of histologically confirmed infective endocarditis, PCR remained negative in a blood culture-negative case for which no bacteria were seen at histological analysis and in one case with visualization of cocci and blood cultures positive for *Enterococcus faecalis*. Ten clinical diagnoses of possible infective endocarditis were ruled out by histopathological analysis of the valves and subsequently by PCR. PCR was negative in 13 of the 14 patients in whom infective endocarditis was rejected on clinical grounds; the other patient was found to have *Coxiella burnetii* infective endocarditis on the basis of PCR and histopathological analysis and was subsequently included in the group of 29 definite cases. In total, PCR contributed to the diagnosis and management of infective endocarditis in 6 of 29 (20%) cases.

Microbiological diagnosis of infective endocarditis is mainly based on blood culture, excised cardiac valve tissue, or infected emboli. This conventional approach is successful in 92 to 95% of cases (5, 13) in which a microorganism is present. Streptococci and enterococci account for 45 to 60% of cases of infective endocarditis (5, 13, 21). They comprise oral viridans streptococci (such as *Streptococcus sanguis*, *Streptococcus salivarius*, and *Streptococcus mutans*) in 17 to 41% of cases (5, 12, 13, 20); intestinal group D streptococci such as *Streptococcus bovis* in 5 to 15% of cases (5, 9) (and up to 25% in recent studies [13]); and enterococci such as *Enterococcus faecalis* in 5 to 10% of cases (5, 13).

Staphylococci represent the second most frequent group associated with infective endocarditis; *Staphylococcus aureus* is recovered in 15 to 23% of cases, and coagulase-negative staphylococci are recovered in 3 to 8% of cases (2, 5, 13). The remaining cases of infective endocarditis are caused by various bacteria such as *Enterobacteriaceae* or fungi such as *Candida* spp.; in addition, some bacteria are more specifically associated with infective endocarditis, such as those of the HACEK group (*Haemophilus parainfluenzae*, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*) in approximately 4% of cases (3, 5, 12, 13).

Conventional cultures are negative in 5 to 8% of cases of infective endocarditis. Some of these cases are due to intracellular bacteria that require special culture conditions, (e.g., *Coxiella burnetii* and *Bartonella* spp., which require cell culture). Detection of specific antibodies is often helpful in such cases (6, 15).

DNA amplification of eubacterium-specific sequences in DNA extracted from cardiac valve tissue is a new tool for etiological diagnosis of infective endocarditis. This approach has so far been used primarily in cases due to fastidious organisms such as *Tropheryma whippelii*, *Coxiella burnetii*, and *Bartonella* spp. (4, 18). Some authors have evaluated molecular diagnosis of infective endocarditis by use of excised valve tissue and blood culture (11, 16). Millar et al. proposed that a positive molecular diagnosis of infective endocarditis be added as a major criterion to Duke's classification (8, 16).

Here, we compared the results of conventional bacteriological and histopathological diagnostic methods for infective endocarditis with those of eubacterial ribosomal DNA (rDNA) amplification and direct sequencing of DNA from excised cardiac valves from 52 patients.

MATERIALS AND METHODS

Samples. In 1999, 52 excised cardiac valves from 52 patients were analyzed at Louis Pradel Hospital, Lyon, France. Thirty-eight of these patients had suspected infective endocarditis (based on clinical, biological, and/or echographic findings), while 14 patients had valve disease, including valvular dystrophies ($n = 8$), prosthesis dysfunctions ($n = 4$), or post-infective endocarditis sequelae ($n = 2$), but no signs of infective endocarditis. The first group of patients comprised 29 men and 9 women with an average age of 58.3 years (range, 29 to 80 years), and

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TABLE 1. Results of blood culture and 16S rDNA PCR assay of valve tissues according to the pre- and postsurgical diagnosis of infective endocarditis

Culture result and PCR diagnosis	No. of cases ^a				Contribution of PCR to diagnosis
	Presurgery suspected (n = 38)		Controls (n = 14)		
	Definite IE	Rejected IE	Definite IE	Rejected IE	
Positive blood culture and identical diagnosis by PCR	21 ^b				Confirmation
Positive blood culture and different diagnosis by PCR	3 ^c				Strong
Negative blood culture and diagnosis by PCR	2 ^d		1 ^e		Strong
Negative blood culture and negative PCR	1 ^f	10		13	None
Positive blood culture and negative PCR	1 ^g				None
Total	28	10	1	13	

^a Infective endocarditis (IE) was either suspected or rejected (controls) presurgery, and the diagnosis was judged definite or rejected after surgery.

^b Four cases due to *Streptococcus bovis*, one due to *Streptococcus pneumoniae*, three due to *Streptococcus sanguis*, *S. oralis*, or *S. parasanguis*, one due to *Streptococcus salivarius*, one due to *Streptococcus mitis*, one due to *Streptococcus dysgalactiae*, three due to *Enterococcus faecalis*, three due to *Staphylococcus aureus*, one due to *Staphylococcus lugdunensis*, one due to *Cardiobacterium hominis*, one due to *Capnocytophaga canimorsus*, and one due to *Sphingomonas sp.*

^c One case each due to *Bartonella henselae* (one positive blood culture with *Pseudomonas aeruginosa*), *Streptococcus mutans* (two positive blood cultures with *Escherichia coli*), and *Streptococcus bovis* (three positive blood cultures with *Streptococcus mutans*).

^d One case due to *Streptococcus bovis* and one due to *Staphylococcus cohnii*.

^e One case due to *Coxiella burnetii*.

^f One case with the valve culture yielding *Staphylococcus epidermidis* (considered a contaminant, as no cocci were seen at histological analysis).

^g One case due to *Enterococcus faecalis*.

the second comprised 10 men and 4 women with a median age of 54.9 years (range, 25 to 79 years). Blood cultures were positive before surgery (range, 2 to 150 days) in 28 patients with suspected infective endocarditis and negative in the other patients.

Excised cardiac valves were jointly examined in a laminar flow unit by a pathologist and a microbiologist. Gross digital photographs were taken. When macroscopic lesions of infective endocarditis were present, the suspect area was divided into three parts for bacteriological, histopathological, and molecular analysis. When infective endocarditis lesions were absent, part of the valve was randomly selected and treated as above.

Histopathological diagnosis. Sections were stained with hematoxylin-eosin-saffran (HES), Gram, Grocott-Gomori methenamine silver, and periodic acid-Schiff stains reagents and then examined independently by two pathologists in concert with a microbiologist. Inflammatory cell infiltration was sought and quantified if present, and the proportion of each cell population (lymphocytes, histiocytes, and polymorphonuclear neutrophils) was determined. Microorganisms were semiquantified after special staining, and their individual and collective morphologies were recorded. Infective endocarditis was classified according to Duke's criteria on the basis of gross features and histopathological findings (8) as definite (active infective endocarditis and/or microorganisms demonstrated by histology in vegetations) or rejected (no pathological evidence of infective endocarditis).

Bacterial culture. Portions of valve tissue were ground with a mortar and pestle and cultured on Columbia blood agar and chocolate agar supplemented with Polyvitex (bioMérieux, Marcy l'Etoile, France) at 35°C for 15 days, aerobically and with 5% CO₂, respectively. In addition, Todd-Hewitt broth supplemented with pyridoxal phosphate was inoculated and incubated for 45 days at 35°C. Direct Gram staining was also performed. Isolated colonies were identified according to standard procedures (17). When conventional cultures remained negative, a sample of valve tissues that had been kept frozen at -80°C was then cultured on human embryonic lung fibroblasts by the French National Reference Center for Rickettsia (D. Raoult, Marseille, France).

DNA extraction. All DNA extraction procedures were carried out in a class II biological safety cabinet (Faster, Ferrara, Italy) in a room (room A, first floor) physically separated from that used to prepare all PCR reagents except DNA (room B, DNA-free, ground floor) and also from that used to prepare nucleic acid amplification mixes (room C, ground floor), and finally from that used for post-PCR analysis (room D, first floor). DNA was extracted from cardiac tissues with the QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. An extraction negative control composed of all reagents used for DNA extraction minus heart tissue was processed in parallel with each sample. Amplification of the human betaglobulin gene served as an internal positive extraction control (see below) (1).

PCR assay. The oligonucleotide primers designed for the 16S rDNA PCR were 91E (5'TCAA[G,T]GAATTGACGGGGGC3') and 13BS (5'GCCCCGGAACGTATTAC3'), which produced a 492-bp fragment of 16S ribosomal DNA (19). Primers PC04 (5'CAACTTCATCCACGTTACC3') and GH20

(5'GAAGAGCCAAGGACAGGTAC3') were used to amplify a 268-bp fragment of the human betaglobin gene (1).

The PCR mixture, which was made up to 50 µl with sterile water (Sigma), contained 1× PCR buffer, MgCl₂ (2.5 mM), 200 µM each deoxynucleoside triphosphate (including dUTP at a dUTP/dTTP ratio of 1:9), 200 µM each primer, 2.5 U of *Taq* DNA polymerase (Eurobio), and 1 U of heat-labile uracil DNA-glycosylase (UNG; Roche) to prevent carryover contamination between PCRs. Five microliters of lysate containing the target DNA was added to the PCR mixture, which was incubated for 10 min at 20°C for U-DNA cleavage by UNG, followed by UNG inactivation by incubation at 94°C for 10 min. PCR was performed for 32 cycles (denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 30 s at 72°C) with a Biometra thermocycler, followed by 10 min of incubation at 72°C. PCR products were analyzed by electrophoresis through a 1.5% agarose gel (Sigma) and sequenced on both strands with PCR primers 91B and 13BS on an automated sequencer at the Genome Express facility.

The 16S rDNA sequences obtained were compared with those available in the GenBank, EMBL, and DDBJ databases with the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov/BLAST/>). Identification to the species level was defined as a 16S rDNA sequence similarity of ≥99% with that of the GenBank prototype strain sequence; identification to the genus level was defined as a 16S rDNA sequence similarity of ≥97% with that of the GenBank prototype strain sequence. A failure to identify was defined as a 16S rDNA sequence similarity of less than 97% with sequences deposited in GenBank at the time of the analysis (7).

RESULTS

In the first group of 38 patients with suspected infective endocarditis, definite infective endocarditis (8) was diagnosed in 28 patients, on the basis of vegetations or intracardiac abscesses found at surgery and histopathologic confirmation of active infective endocarditis. Valve cultures was positive in 10 cases and yielded the same bacterial species as that found by blood culture in 9 of these cases (in one case the valve culture yielded *Staphylococcus epidermidis*, which was considered a contaminant; see below). The median duration of antibiotic treatment before surgery was 31.5 days (range, 8 to 150 days) when the valve culture was negative and 9.5 days (range, 2 to 35 days) when the valve culture was positive.

Histological analysis in cases of definite infective endocarditis showed cocci in 25 cases, bacilli in two cases, and no bacteria in one case. In 21 of these 28 patients, PCR results

obtained with the excised valve matched those of blood culture (Table 1). PCR results disagreed with the initial bacteriological diagnosis in three cases: (i) in a case in which a single blood culture yielded *Pseudomonas aeruginosa*, *Bartonella henselae* infective endocarditis was diagnosed (confirmed by serology; no bacteria were seen at the Gram stain); (ii) in a case with repeated blood cultures positive for *Escherichia coli*, PCR identified *Streptococcus mutans*; the latter species had been responsible for another episode of infective endocarditis 18 months previously (cocci were seen on the Gram-stained valve tissue); and (iii) in a case in which the blood culture yielded *Streptococcus mutans*, PCR identified *Streptococcus bovis*. PCR was negative in a case in which two blood cultures were positive for *Enterococcus faecalis*.

The interval between the start of antibiotic treatment and surgery was 34 days; cocci were seen on Gram-stained valve tissue, but PCR remained negative despite three separate DNA extraction and amplification procedures. PCR was also negative in another case of definite infective endocarditis with a negative blood culture in which the valve culture yielded *Staphylococcus epidermidis*; the latter was considered a contaminant, as no cocci were seen at histological analysis. In two patients with a negative blood culture, the etiological agent of infective endocarditis was identified by PCR on excised valve tissue: (i) one case was attributed to *Streptococcus bovis* in a patient who had received antibiotic treatment before blood sampling; cocci were seen on the Gram-stained valve tissue; (ii) another case was attributed to *Staphylococcus cohnii*; it involved an intravenous drug user who had been treated with a cephalosporin before blood sampling; histopathological analysis of the excised valve showed the presence of cocci.

In 10 cases of clinically suspected infective endocarditis (no diagnosis of definite endocarditis according to the Duke criteria), the diagnosis of infective endocarditis was rejected on the basis of histopathologic findings. PCR amplification of DNA from the excised valves was negative, although very weak bands were observed in two cases (sequence data showed that they were environmental contaminants). Blood culture was negative in six of these ten patients, while coagulase-negative staphylococci grew from one sample from each of the other four patients.

The diagnosis of infective endocarditis was rejected in all but one of the 14 negative control patients. PCR identified *Coxiella burnetii* infective endocarditis in one of these patients, and this was confirmed by serological tests (by immunofluorescence, immunoglobulin G [IgG] phase I, 1/51,200; IgG phase II, 102,400) and immunohistologic analysis with an anti-*Coxiella burnetii* monoclonal antibody, and valve tissue grew *Coxiella burnetii* on human embryonic lung fibroblasts (serology, immunohistology, and culture done at the French National Reference Center for Rickettsia, Marseille, France).

DISCUSSION

We evaluated molecular diagnosis targeting eubacterium-specific sequences on cardiac valves from 29 patients with histologically confirmed infective endocarditis and 23 patients for whom a diagnosis of infective endocarditis was rejected. PCR findings usually agreed with the results of conventional bacteriological and histopathologic diagnosis. Bacteria were

visualized in valve tissue for up to 150 days after initial antibiotic treatment and were always associated with histopathological evidence of active infective endocarditis. PCR was positive even when the valve tissue culture was negative.

In one patient, only one of six blood cultures yielded *Streptococcus salivarius*; histology revealed active infective endocarditis (cocci), and PCR established the presence of *S. salivarius* DNA in the heart valve. PCR was the only method to provide the etiological diagnosis in two cases (Table 1); this led to the prescription of appropriate antibiotic treatment, together with colonoscopy in the patient with *Streptococcus bovis* infective endocarditis. Goldenberger et al. (11) used a similar molecular approach to study 18 cases of infective endocarditis and also identified the causative agent in two cases in which conventional blood culture was negative.

In our study, PCR modified the initial bacteriological diagnosis in three cases. In the first, *Streptococcus bovis* was identified by PCR (instead of *Streptococcus mutans*); the initial isolate was not referred to our laboratory for phenotypic identification, but colonoscopy showed a colonic villous tumor. In the second, *Streptococcus mutans* was identified by PCR in a patient who had had *Streptococcus mutans* infective endocarditis 18 months previously and currently had interfering *Escherichia coli* sepsis (10). In the third, *Bartonella henselae* was identified in a patient with interfering *Pseudomonas aeruginosa* sepsis secondary to needle biopsy of the kidney. A definite case of *Coxiella burnetii* infective endocarditis was fortuitously diagnosed by PCR, confirming the potentially mild clinical and biological signs of early-stage *C. burnetii* infective endocarditis (14, 15). This case underlines the need for careful investigation of all patients undergoing valve replacement surgery, whatever their initial diagnosis. In total, PCR contributed to the etiologic diagnosis of infective endocarditis in 6 of 29 cases (20%).

PCR positivity due to contamination occurred in only 3 of the 23 cases of rejected infective endocarditis. It yielded sequences related to environmental bacteria not considered responsible for infective endocarditis. Moreover, histopathological analysis did not reveal the presence of bacteria in these cases. Overall, DNA contamination did not interfere with our PCR results, probably because of the large number of bacteria present in resected tissue from patients with infective endocarditis and the strict measures taken to prevent microbial contamination and carryover contamination between PCRs. This is in keeping with previous reports in which similar precautions and controls were used (11, 16).

PCR was always positive when bacteriological culture of the same tissue fragment was also positive and when bacteria were visualized by histopathological analysis except for one case of *Enterococcus faecalis* infective endocarditis. The negative PCR result in this case may have been due to three factors: the long interval between antibiotic treatment and surgery (34 days); the use of an inappropriate valve fragment for PCR; or the presence of PCR inhibitors (although extraction and amplification controls gave the expected results, and amplification of the beta-globin gene was positive).

In conclusion, we confirm that broad-range PCR amplification followed by direct sequencing is a reliable and accurate method when applied to resected heart valves and that it can be used as an adjunct to culture methods. In the view of Millar et al. (16), PCR should only be used in precise clinical cases of

culture-negative infective endocarditis. However, our results suggest that this molecular approach may prove useful in other cases: (i) when bacteria rarely associated with infective endocarditis are recovered by blood culture (e.g., *Enterobacteriaceae*); (ii) when blood culture is positive only once (e.g., our case of *Streptococcus salivarius* infective endocarditis); (iii) when strain identification is unsure (e.g., our case of *Streptococcus mutans*/*Streptococcus bovis* infective endocarditis); and (iv) when valve replacement for noninfectious indications leads to histologic diagnosis of infective endocarditis.

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