MAJOR ARTICLE

Etiologic Diagnosis of Infective Endocarditis by Broad-Range Polymerase Chain Reaction: A 3-Year Experience

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We analyzed surgically resected endocardial specimens from 49 patients by broad-range PCR. PCR results were compared with (1) results of previous blood cultures, (2) results of culture and Gram staining of resected specimens, and (3) clinical data (Duke criteria). Molecular analyses of resected specimens and previous blood cultures showed good overall agreement. However, in 18% of patients with sterile blood cultures, bacterial DNA was found in the resected materials. When data from patients with definite or rejected cases of infective endocarditis (IE) were included, the sensitivity, specificity, and positive and negative predictive values of broad-range PCR were 82.6%, 100%, 100%, and 76.5%, respectively, overall, and 94.1%, 100%, 100%, and 90%, for cases of native valve endocarditis. The sensitivity, specificity, and positive and negative predictive values of culture of resected specimens from patients with native valve endocarditis were 17.6%, 88.9%, 75%, and 36.4%. We recommend broad-range PCR of surgically resected endocardial material in cases of possible IE, in cases of suspected IE in which blood cultures are sterile, and in cases in which organisms grow in blood cultures but only Duke minor criteria are met. We propose to add molecular techniques to the pathologic criteria of the Duke classification scheme.

Infective endocarditis (IE), infection of the endocardial surface, is a rare but severe disease. In Europe, the incidence is ~6–7 cases/100,000 person-years [1, 2], and the overall mortality rate is 20%–25%, depending on the causative microorganism and the presence of complications and comorbidities [3]. IE is usually diagnosed according to the Duke scheme, by clinical or pathologic criteria (i.e., histologically confirmed pathologic lesions or demonstration of a causative microorganism by culture or histologic examination in a sample from a vegetation, an embolism, or an intracardiac abscess) [4, 5]. The Duke clinical criteria include major criteria (i.e., blood culture positive for IE, echocardiographic findings consistent with IE, and valvular regurgitation) and minor criteria (i.e., predisposition, fever, vascular and immunologic phenomena, and growth of organism in blood cultures but failure to meet a major criterion). In large series, blood cultures were sterile in 2.5%–31% of cases of IE [6]. Blood cultures often are sterile as a result of previous antibiotic therapy [1] or because of fastidious and difficult-to-cultivate microorganisms such as HACEK group bacteria, *Bartonella* species, *Coxiella burnetii, Legionella* species, *Mycoplasma* species, and *Tropheryma whipplei* [7, 8]. In such cases, the etiologic agent may be identified by serologic and/or immunohistologic techniques [9]. Recent case reports have shown the usefulness of broad-range amplification of

Received 20 November 2002; accepted 3 March 2003; electronically published 9 July 2003.

Financial support: University of Zurich.

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Clinical Infectious Diseases 2003; 37:167–72

1058-4838/2003/3702-0002$15.00

16S rDNA (referred to as "broad-range PCR") for identification of the causative agent of IE [10–14]. However, only a few studies so far have addressed the utility of nucleic acid amplification techniques for diagnosis of IE in a systematic fashion [15, 16].

For >4 years, we have used broad-range PCR as part of our microbiological workup procedure for endocardial specimens [15]. We here report a retrospective study that included 49 patients who were referred for endocardial surgery during January 1998 through March 2001. Our main focus was to compare the results of broad-range PCR with those of standard microbiological procedures, to evaluate the clinical utility of broad-range PCR for the diagnosis of IE, and thus to suggest possible guidelines for the use of broad-range PCR in clinical practice.

PATIENTS AND METHODS

Patients. From January 1998 through March 2001, all patients ($n = 49$) from a tertiary health care center in Zurich (University Hospital of Zurich) who had been referred for endocardial surgery and from whom specimens had been submitted to the Institute of Medical Microbiology (University of Zurich) were included in this study. Clinical and pathologic data, including blood culture results for up to 6 months before surgery, were collected from the medical records, and cases were classified according to the modified Duke criteria [5]. Forty-two patients (86%) were male, and 7 (14%) were female; the mean age ($\pm$ SD) was 55 years ($\pm$ 17 years; range, 10–83 years). Twenty-two patients had definite IE and 13 patients had possible IE according to the modified Duke criteria [5]; in 14 patients, surgery was performed because of valvular dysfunction (IE was excluded on the basis of the Duke criteria). Eighteen patients (37%) had prosthetic valves before surgery. A total of 63 specimens were obtained and analyzed, 52 of which were valves (38 aortic valves and 14 mitral valves) and 11 of which were of other materials, such as aortic vessels ($n = 7$), swabs from the aortic valve ($n = 3$), and a vegetation on a pacemaker ($n = 1$). In 39 patients, blood samples were obtained for culture before surgery. For these patients, on average 6.9 blood cultures (range, 1–25 cultures; each blood culture consisted of 1 aerobic and 1 anaerobic bottle) were done. Eleven additional patients previously had had IE and underwent surgery because of valvular dysfunction (anti-infective therapy had been completed between 8 days and 8.5 years before surgery).

Cultures. Cultures were performed as described elsewhere [15]. Briefly, aerobic and anaerobic blood cultures (BacT/Alert) were incubated at 37°C for at least 6 days. If growth was found in blood cultures, aliquots were subcultured on Columbia sheep blood agar (Difco) and on brucella sheep blood agar (Becton Dickinson BBL). Single blood cultures positive for *Propionibacterium acnes* or coagulase-negative staphylococci were rated as contaminated. Parts of endocardial specimens were ground, prepared for Gram staining, and cultured aerobically and anaerobically on Columbia sheep blood agar, chocolate agar (GC II Agar base with 1% hemoglobin and 1% IsoVitaleX; BBL), and *Brucella* sheep blood agar (BBL) for 2–3 days at 37°C. In addition, pieces of valve tissue were inoculated into thioglycolate medium (BBL) and incubated for 10 days.

DNA extraction and broad-range PCR. DNA extraction and broad-range PCR were performed essentially as described elsewhere [15]. In brief, small pieces of tissue were digested with proteinase K in the presence of 0.5% SDS. DNA was purified with the QIAamp Blood Kit (Qiagen), resulting in a sample volume of 100 μL. One and 5 μL of the DNA extract were used for amplification in a total volume of 50 μL containing 1.25 U of AmpliTaq DNA polymerase, LD (Applied Biosystems). Part of the 16S rDNA (positions 10–806, according to *Escherichia coli* numbering [17]) was amplified with primers BAK11w (5′-AGTTTGATC[A/C]TGGCTCAG) and BAK2 (5′-GGACTAC[A/C/T]AGGGTATCTAAT) for 40 cycles. Insulin-like growth factor II amplification with primers IGF-P2 (5′-CTTGGACTTTGAGTCA) and IGF-P3 (5′-GGTCGTG-CCAATTACA) was used as inhibition control.

The amplicons were examined by high-resolution polyacrylamide gel electrophoresis (CleanGel; Pharmacia) in which silver staining was used. Positive samples were recognized by a single prominent band of appropriate size; negative samples were those that generated a characteristic banding pattern with several bands of smaller size (i.e., 100–200 bp instead of 800 bp) and low intensity. Bands of proper size were excised and reamplified (positions 10–533, according to *E. coli* numbering) with primers BAK11w and BAK533r (5′-TTACCGCGGCTGCTGG-CAC) for 40 cycles using 1.25 U of *Taq* DNA polymerase (Amersham Biosciences) [15]. For reamplification, a piece of the excised band was added directly to the reamplification mix without prior purification of the DNA. To prevent carry-over contamination, we used physically separate rooms for sample preparation, setup of PCR mixes, nucleic acid amplification, and analysis of PCR products. In addition, several negative and positive controls for DNA extraction and amplification were used.

Occasionally, contaminating DNA was observed both in negative controls and in patient samples. Although the contaminating DNA produced a band that was similar in size to that seen in positive samples when high-resolution polyacrylamide gel electrophoresis was done, this was always less prominent than in the case of true-positive samples. Sequence analyses demonstrated that this reagent contamination derived from waterborne *Pseudomonas* species.

Sequence analysis. The nucleic acid sequence of PCR-amplified gene fragments was determined using automatic DNA sequencing (ABI Prism 310 Genetic Analyzer [Applied

Table 1. Results of broad-range PCR compared with results of culture and microscopic examination of endocardial specimens.

Specimen status	Results of broad-range PCR, no. of specimens	
	Positive (n = 25)	Negative (n = 38)
Culture-positive, microscopy-negative	3[a]	3[b]
Culture-negative, microscopy-positive	7[c]	1[d]
Culture-positive, microscopy-positive	0	0
Culture-negative, microscopy-negative	15	34

[a] In 1 case, results were contradictory; viridans streptococci were recovered on culture, but broad-range PCR yielded a sequence with 100% identity with 3 different *Staphylococcus* species *(S. arlettae, S. capitis,* and *S. capprae).*

[b] Two samples showed growth of *Propionibacterium acnes* (samples were from 2 swabs of the aortic valve from the same patient). Coagulase-negative staphylococci grew on culture of a sample from another patient after enrichment in liquid medium. These 2 patients did not have any other clinical or pathologic evidence of infective endocarditis, and therefore the PCR results were considered to be true negative.

[c] In all 7 microscopy-positive samples, PCR results were congruent (i.e., both diagnostic procedures revealed gram-positive cocci).

[d] The results of both PCR and microscopic examination of a second sample from the same patient were positive.

Biosystems] and ALF automatic DNA sequencer [Pharmacia]). Database analysis was done in a 2-step procedure. A first search was performed with the FASTA algorithm of the GCG Wisconsin software package (Accelrys). All positions showing differences from the best scoring reference sequence were visually inspected in the electropherogram, and the sequence was corrected if necessary. Then a second search was done using BLAST (http://www.ncbi.nlm.nih.gov/blast). Undetermined nucleotides in the determined sequence or the reference sequence were counted as matches. For identification to the genus or species level, the following criteria were used: 0–2 mismatches with the sequence entry of an established species was interpreted as species identification, and 0–2 mismatches with the sequence entry of an unclassified species or >2 mismatches with the highest-score entry was interpreted as genus identification.

RESULTS

Broad-range PCR versus culture and microscopic examination of resected specimens. In 2 of the 63 specimens we investigated, PCR was inhibited (in these patients, cultures of previously obtained blood samples were sterile). The results of broad-range PCR were positive in 25 specimens from 23 patients, and 15 of these specimens were culture and microscopy negative (table 1). Five sequences were identified at the genus level, and 20 sequences could be assigned to a species. Three samples were culture positive and PCR negative; in these cases, the culture results were considered to be false positive (table 1).

Broad-range PCR versus blood culture. Of the 39 patients from whom blood samples were obtained for culture before surgery (mean number of blood cultures, 6.9; range, 1–25 cultures), 22 had at least 1 positive blood culture (mean number of positive blood cultures, 5.8; range, 1–19 cultures) (table 2). The results of PCR in samples from 19 of these 22 patients were positive (table 3). For 13 patients (68%), both procedures resulted in identification of the same species; for 6 (32%) of the 19 patients, PCR yielded a more precise result (e.g., *Streptococcus salivarius* instead of viridans streptococcus, or an unclassified oral *Streptococcus* species instead of *Streptococcus oralis* or *Streptococcus sanguis*). In the latter case, the obtained sequence exactly matched that of an unclassified oral *Streptococcus* species sequence but exhibited <98% homology to the most closely related established species, *S. sanguis.* The 3 patients for whom blood culture results were positive but PCR results were negative had definite IE, according to the Duke criteria; the PCR results were considered to be false negative (table 2).

For 3 patients, the results of PCR were positive, even though the results of blood cultures (at least 2 pairs), microscopic examination, and culture of endocardial specimens were negative (table 2). In all 3 cases, the results of PCR were considered to be true positive. For 2 of these patients, IE was diagnosed as "possible," according to the Duke criteria; PCR found *Haemophilus aphrophilus* in one case and *Streptococcus bovis* in the other. Both patients were receiving antibiotics at the time that

Table 2. Results of broad-range PCR in endocardial specimens compared with cultures of previously drawn blood samples.

Blood culture results	No. of patients (n = 49)	Results of broad-range PCR, no. of patients	
		Positive (n = 23)	Negative (n = 26)
Positive for any organism	22	19	3
Fulfilled Duke major criteria[a]	18	17	1[b]
Did not fulfill Duke major criteria for IE but did fulfill Duke minor criteria	4	2	2[c]
No organisms grown on culture	17	3[d]	14
Not done	10	1	9

NOTE. IE, infective endocarditis.

[a] Microorganism typical of IE found in at least 2 separate blood cultures or microorganism consistent with IE found persistently in blood cultures

[b] Patient had definite IE, according to Duke criteria (*Staphylococcus aureus* was found in 7 of 7 blood cultures). Results of PCR were negative, although antibiotic therapy was started only 4 days before surgery (false-negative PCR).

[c] Includes 1 patient with coagulase-negative staphylococci in 3 of 10 blood cultures and 1 patient with *Propionibacterium acnes* in 7 of 21 blood cultures. PCR results were considered to be false negative in both cases.

[d] PCR results were considered to be true positive; for more details, see Results.

Table 3. Organisms identified by blood culture and broad-range PCR in samples from 19 patients with infective endocarditis for whom both methods had positive results.

Bacterial group, patient	Identification by broad-range PCR	Identification by blood culture
Viridans streptococci		
1	*Streptococcus australis*	*Streptococcus mitis*
2	*Streptococcus bovis*	*S. bovis*
3	*S. bovis/gallolyticus*	*S. bovis*
4	*Streptococcus gordonii*	*S. gordonii*
5	*S. mitis*	*S. mitis*
6 and 7	*Streptococcus mutans*	*S. mutans*
8	*Streptococcus salivarius*	Viridans streptococcus
9 and 10	Unclassified oral streptococcus	*S. mitis*
11 and 12	Unclassified oral streptococcus	*Streptococcus oralis/sanguis*
Enterococci		
13 and 14	*Enterococcus faecalis*	*E. faecalis*
Staphylococci, 15–18	*Staphylococcus aureus*	*S. aureus*
Haemophilus species, 19	*Haemophilus parainfluenzae/paraphrophilus*	*H. parainfluenzae*

blood samples (10 and 3 pairs) were obtained for culture. In the third patient, *T. whipplei* was identified by PCR. This patient underwent surgery for aortic insufficiency. The valvular pathology, described as "lymphoplasmacellular inflammation with formation of granulomas and fibrosis," was interpreted as sterile endocarditis in a patient with seronegative spondylarthritis. However, 2 different PCRs specific for *T. whipplei* (targeting a different part of the 16S rDNA and the ribosomal intergenic spacer region [18, 19]) were performed on aortic material, confirming the results of broad-range PCR. These findings make it unlikely that laboratory contamination occurred, because for each PCR, a specific positive control is used that only includes the region of interest. From these data, we concluded that *T. whipplei* most probably was the true cause of IE in this case (true-positive PCR result). The patient has not yet shown other manifestations of Whipple disease. Cases of Whipple endocarditis in the absence of overt gastrointestinal disease [11] or without other signs of Whipple disease have been reported elsewhere [10, 20].

Sensitivity, specificity, and positive and negative predictive values of assays. According to the Duke criteria, which include clinical and pathologic features, 22 patients were considered to have definite IE; 13 patients, possible IE; and 14 patients, no ("rejected") IE. The patient with Whipple endocarditis was considered to have definite IE, resulting in the inclusion of 23 patients with definite IE and 13 patients with rejected IE in our study. Broad-range PCR results were positive for 19 of 23 patients with definite IE, 4 of 13 patients with possible IE, and 0 of 13 patients with rejected IE (table 4).

Definite and rejected cases of IE were used as the reference standard for calculating the sensitivity and specificity of broad-range PCR. Overall, sensitivity was 82.6%, specificity was 100%, positive predictive value was 100%, and negative predictive value was 76.5%. Six patients with definite IE, 8 patients with possible IE, and 4 patients with rejected IE had prosthetic valves. Broad-range PCR was positive in samples from 16 of 17 of the patients with definite native valve endocarditis (NVE) but only in 3 of 6 of the patients with definite prosthetic valve endocarditis (PVE). The sensitivity, specificity, and positive and negative predictive values of broad-range PCR were 94.1%, 100%, 100%, and 90%, respectively, when the analysis was limited to cases of NVE and 50%, 100%, 100%, and 42.9% when the analysis was limited to cases of PVE.

Organisms grew on culture of resected specimens from 3 of 23 patients with definite IE (3 of 17 patients with NVE), 0 of 13 patients with possible IE, and 2 of 13 patients with rejected IE (1 of 9 cases with NVE and 1 of 4 cases with PVE). The sensitivity, specificity, and positive and negative predictive values for culture of resected specimens were 13%, 84.6%, 60%, and 35.5%, respectively, overall, and 17.6%, 88.9%, 75%, and 36.4% when the analysis was limited to cases of NVE. The failure to obtain a positive result for specimens from any of the patients with PVE makes statistical analysis irrelevant but indicates the limited use of culture techniques in this context.

Patients with previous IE. Eleven additional patients previously had had IE. Adequate anti-infective therapy had been completed between 8 days and 8.5 years before surgery. The patients underwent surgery because of valvular dysfunction in the absence of acute signs of endocarditis. In 6 patients, the results of PCR were positive. In these cases, diagnosis was made 1, 2, 4, 7, and 23 months before surgery. Two of these patients were intravenous drug users who had multiple episodes of IE >6 months before surgery. The cultures of blood samples from both patients were positive for *Staphylococcus aureus* and *Strep-*

Table 4. Comparison of culture and broad-range PCR in patients with infective endocarditis (IE) according to the Duke classification scheme.

Duke category	Total no. of patients (n = 60)	No. of patients with positive results of culture		No. of patients with positive results of PCR (n = 60)
		Blood sample (n = 49)	Resected specimen (n = 60)	
Definite IE	23	20[a]	1	19
Possible IE	13	2	0	4
Rejected IE	13	0[a]	3[b]	0
Previous IE[c]	11	5[a]	0	6

[a] Blood cultures were done for only 22 patients with definite IE, 4 with rejected IE, and 10 with previous IE.

[b] These 3 patients did not have any other clinical or pathologic evidence of IE; culture results were considered to be false positive. Cultures were done for only 13 patients.

[c] Anti-infective therapy was suspended for a period ranging from 8 days to 8.5 years before surgery.

tococcus dysgalactiae (in 1 patient, *Streptococcus pyogenes* was also found). In both cases, histologic examination revealed chronic inflammation; PCR in resected specimens identified *S. dysgalactiae* (isolated in blood cultures from these 2 patients 7 and 23 months before valve replacement).

DISCUSSION

In this study, we used a combination of broad-range PCR of 16S rDNA and nucleic acid sequencing to identify the causative agent of IE. We analyzed all patients of a tertiary care hospital who were referred for endocardial surgery during a 3-year period and from whom specimens were submitted to our laboratory. In addition to patients with definite or possible IE, patients with no suspicion for IE and patients with a previous episode of IE were included in this study.

Our study demonstrates that broad-range PCR is a useful tool for the analysis of resected heart valves. For the majority of PCR-positive specimens, no organisms were found by culture on standard media or by microscopic examination. This is probably because 90% of the patients had received antibiotics before surgery. PCR results were in strong agreement with the results of culture of previously obtained blood samples, and sequencing provided more-accurate results in 33% of cases (tables 2 and 3). However, imprecise identification by traditional techniques did not affect management of patients. Nonetheless, for epidemiological reasons, a precise identification of the causative agent is important.

IE usually is diagnosed by the Duke criteria, which have been accepted by most clinicians [4]. The Duke criteria build on the earlier published Beth Israel criteria [21] by including echocardiographic findings and the isolation of typical IE pathogens from blood cultures as major criteria for a clinically definite categorization of IE. Further modification was proposed by Li et al. [5] and Fournier et al. [22]. On the basis of our experience, we propose that nucleic acid amplification–based detection and identification of IE pathogens from endocardial specimens should be included in the Duke criteria (i.e., a microorganism can be demonstrated by culture, histologic examination, or molecular-based techniques to fulfill a pathologic criterion and thus to contribute to the definite diagnosis of IE). This proposal is supported by the fact that PCR is far more sensitive and specific than culture of resected specimens, a procedure which is accepted as a pathologic criterion. More recently, Millar et al. [16] suggested that molecular-based diagnosis of IE using blood cultures be included as an additional major criterion within the Duke clinical criteria. In this study, the authors used PCR to identify DNA extracted from blood cultures and showed the feasibility of identifying the causative agent in cases of blood culture–negative IE [16].

The present study indicates that, if broad-range PCR of resected specimens is included among the pathologic Duke criteria, cases that otherwise were classified as possible IE may be classified as definite IE, regardless of the result of culture of previously obtained blood samples. Four of 13 patients with possible IE in this study would have been classified as having definite IE on the basis of a positive PCR result. For the other 9 patients in this group, the negative PCR result did not exclude IE. The clinical and pathologic investigations performed in this study did not allow strict definition of whether these patients had or did not have IE, and therefore those cases were classified as "possible IE." Blood culture results that fit the Duke minor criteria often are associated with some uncertainty; PCR results from endocardial specimens may verify the causative agent and aid the clinician in interpretation of culture findings. Broad-range PCR may be helpful for cases of so-called blood culture–negative IE [11, 23, 24]. In the present study, PCR made it possible to diagnose and identify the cause of IE for 23% of the patients with definite or possible blood culture–negative IE.

The sensitivity, specificity, and positive and negative predictive values of broad-range PCR were calculated to be 82.6%, 100%, 100%, and 76.5%, respectively. When only cases of NVE were included in the analysis, the sensitivity and negative predictive value increased to 94.1% and 90%, respectively. In spite of the excellent specificity, a positive PCR result always has to be interpreted within the clinical context; even several months after therapy for IE has been completed, PCR results may still be positive. The low sensitivity of 50% found when only cases of PVE are included in the analysis highlights the difficulty of obtaining the correct piece of specimen—if mechanical prosthetic devices were collected, pieces of adjacent tissue had to be used for digestion and DNA extraction, and this tissue is not necessarily involved in infection [25].

On the basis of the findings of the present study, we propose to perform broad-range PCR in resected endocardial specimens (1) in cases of possible IE, (2) in cases of blood culture–negative suspected IE, and (3) in cases of growth in blood cultures that fulfills the Duke minor criteria to verify the causative agent of IE. Because of the limited availability of these highly specialized molecular investigations, specimens should be sent to a laboratory with experience in these techniques. The present data also indicate that nucleic acid amplification techniques do not yield additional information in cases of definite IE that fulfill a Duke major criterion and in which microorganisms grow on blood cultures or in cases with no evidence of IE. A limitation of broad-range PCR is that it does not provide information about antimicrobial susceptibility. Thus, routine culture and susceptibility testing remain important cornerstones of the microbiological diagnosis of IE.

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