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Two-step bacterial broad-range polymerase chain reaction analysis of heart valve tissue improves bacteriological diagnosis of infective endocarditis ☆

Rémi Boussier [a], Sylvie Rogez [a], Bruno François [b], Eric Denes [c], Marie-Cécile Ploy [a], Fabien Garnier [a,*]

[a] CHU Limoges, Laboratoire de Bactériologie-Virologie-Hygiène, 87800 Limoges, France
[b] CHU Limoges, Service de Réanimation Polyvalente, 87800 Limoges, France
[c] CHU Limoges, Service de Maladies Infectieuses, 87800 Limoges, France

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ABSTRACT

Positive heart valve (HV) culture is a major Duke's criterion for the diagnosis of infective endocarditis but is poorly sensitive. Two broad-range 16S rDNA polymerase chain reaction (PCR) methods were applied to 31 HV samples: first, a real-time method, then conventional end-point PCR was applied to HV samples on which the first PCR was negative. Five specific real-time PCR procedures were also used in order to identify *Bartonella* spp., *Tropheryma whipplei*, *Chlamydophila pneumoniae*, *Mycoplasma pneumonia*, and *Coxiella burnetii*. A strategy combining the 2-step broad-range PCR methods improved the sensitivity of the molecular method from 38.7% to 58%. Specific PCR identified 1 *T. whipplei*, which was also identified by conventional end-point PCR. These results confirm that blood culture is the gold standard for the diagnosis of infective endocarditis, shows that molecular methods applied to HV can be useful when blood culture is negative, and that 2-step broad-range PCR approach seems to be more sensitive.

1. Introduction

First described by Osler in 1885 (Osler, 1885), infective endocarditis (IE) is a life-threatening disease with an annual incidence of 31 cases per million inhabitants in France (Hoen et al., 2002) and a mortality rate of about 20% (Hoen et al., 2002). The incidence of IE has remained quite stable in recent decades (Hoen et al., 2002), but the microbiological profile has evolved, with an increasing proportion of group D streptococci and staphylococci (Fournier et al., 2010; Moreillon and Que, 2004; Prendergast, 2006). IE is often fatal without antibiotic therapy, and surgery is sometimes necessary. Rapid identification of the causative pathogen is thus crucial.

IE is usually defined by clinical, histologic, and/or microbiological criteria, using Duke's criteria (Fournier et al., 2010; Li et al., 2000). Bacteremia is a consistent feature of IE, and blood culture is therefore the gold standard for bacteriological diagnosis of IE (Li et al., 2000). However, blood cultures are negative in 2.5% to 31% of cases (Houpikian and Raoult, 2005; Moreillon and Que, 2004). When blood culture is negative, alternative diagnostic methods include serology (Fournier et al., 2010; Houpikian and Raoult, 2005; Raoult, 2006), immunohistology (Fournier et al., 2010; Lepidi et al., 2006; Morris et al., 2003), pathologic examination, and culture of heart valve (HV) tissue. Surgical HV excision is required in 25% to 42% of cases (Moreillon and Que, 2004), and HV culture positivity is a Duke's major diagnostic criterion (Li et al., 2000). However, HV culture can be falsely positive due to contamination between resection and laboratory processing (Lang et al., 2004; Muñoz et al., 2008), and falsely negative because of prior antibiotic therapy (Lang et al., 2004) or the involvement of fastidious or nonculturable bacteria (Fournier et al., 2010). In this context, molecular methods applied directly to HV tissue have shown promise in negative-culture IE, and sequencing of the gene encoding 16S ribosomal RNA (rRNA) is an accurate means of identifying microorganisms (Podglajen et al., 2003) and, offers sensitive and rapid diagnosis (Fournier et al., 2010; Gauduchon et al., 2003; Goldenberger et al., 1997; Houpikian and Raoult, 2005; Marín et al., 2007; Podglajen et al., 2003). Here we applied a 2-step broad-range polymerase chain reaction PCR (16S rDNA target) and a specific real-time PCR to HV samples and compared the results with those of serologic tests and conventional microbiological methods in order to evaluate the clinical utility of PCR for etiologic diagnosis of IE. We propose an algorithm for PCR use in clinical practice.

2. Materials and methods

2.1. Patients

This study involved HV samples excised at Limoges Teaching Hospital, France, between January 1, 2002, and December 31, 2007,

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* Corresponding author. Tel.: +33-5-55-05-63-48; fax: +33-5-55-05-67-22.
E-mail address: fabien.garnier@unilim.fr (F. Garnier).

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from 31 patients with definite endocarditis based on Duke's modified criteria (Fournier et al., 2010).

The aortic valve was affected in 15 cases and the mitral valve in 16 cases. All HVs were native.

2.2. Molecular microbiology

2.2.1. DNA Extraction

Fresh HVs were examined and portions with fibrotic lesions were disrupted in sterile conditions and stored at −80 °C. Each portion was disrupted and placed in a Lysing Matrix A® tube (MPBiomedicals, Illkirch, France) for mechanical lysis with FastPrep FP 120® (MPBiomedicals) followed by chemical lysis and DNA extraction with the QIAamp DNA Mini Kit® (Qiagen, Courtaboeuf, France). DNA extracts were stored at −80 °C until use.

2.2.2. Broad-range real-time PCR

Broad-range real-time PCR (RT-PCR) targeting part of the 16S rRNA gene was applied to all HV DNA extracts, using primers 91E and 13BS (Table 1), yielding a 492-bp fragment (Gauduchon et al., 2003). The PCR reaction mix (25 μL) contained nuclease-free water, 10 pmol of each primer, 12.5 μL of SYBR Premix Ex Taq® (Takara, Orgentec SA, Trappes, France), and 5 μL of DNA extract. RT-PCR was performed in a SmartCycler II® instrument (Cepheid, Europe SA, Maurens-Scopont, France) with preincubation at 95 °C for 5 s to activate the enzyme, and 40 thermal cycles as follows: 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 20 s. Amplification of the human betaglobulin gene with primers β-Glo 1 and β-Glo 2 (Table 1) in the same PCR conditions served as a positive extraction control.

2.2.3. Broad-range end-point PCR

When 16S rDNA real-time PCR amplification failed, end-point PCR (EP-PCR) was performed with primers 1510/1492 and 8–27 (Relman et al., 1992) (Table 1) to amplify the entire 16S rRNA gene (approximately 1.5 kb). The PCR mixture, made up to 50 μL with sterile water, contained 10× PCR buffer (Qiagen), 25 mmol/L $MgCl_2$ (Qiagen), 0.2 mmol/L each of deoxyribonucleoside triphosphate (dATP, dCTP, and dGTP), 0.4 mmol/L dUTP (Roche, Diagnostics, Mannheim, Germany), 10 pmol of each primer, 2 U of *Taq* DNA polymerase (Qiagen), and 0.5 U of heat-labile uracil DNA-glycosylase (UDG) (Roche) to prevent carryover contamination between PCRs. Five microliters of purified DNA was added to the PCR mixture, then incubated for 10 min at 37 °C for U-DNA cleavage by UDG, followed by UDG inactivation by incubation at 94 °C for 3 min. PCR was performed for 35 cycles, as follows: denaturation for 30 s at 94 °C, annealing for 60 s at 59 °C, and extension for 60 s at 72 °C in a GeneAmp PCR System 2400® (Perkin Elmer Applied Biosystems, Courtaboeuf, France), followed by 10 min at 72 °C.

Table 1
Oligonucleotide primers used for PCR amplification.

Primer	Oligonucleotide sequence (5′-3′)	Target	Reference
91E	TCAAA(GT)GAATTGACG GGGGC	16S rDNA gene	Gauduchon et al., 2003
13BS	GCCCGGGAACGTATTAC	16S rDNA gene	Gauduchon et al., 2003
1510/1492	GGTTACCTTGTTACGACTT	16S rDNA gene	Relman et al., 1992
8/27	AGAGTTTGATCCTGGCTCAG	16S rDNA gene	Relman et al., 1992
β-Glo 1	ACACAACTGTGTGTTCACTAGC	Betaglobulin	This study
β-Glo 2	CAACTTCATCCACGTTCACC	Betaglobulin	This study
BhCS.781p	GGGGACCAGCTCATGGTGG	*Bartonella*	Norman et al., 1995
BhCS.1137n	AATGCAAAAAGAACAGTAAACA	*Bartonella*	Norman et al., 1995
53.3F	AGAGAGATGGGG GCAGGAC	*T. whipplei*	Fenollar et al., 2004
53.3R	AGCCTTTGCCAGACAGACAC	*T. whipplei*	Fenollar et al., 2004
Cp522s	TGCTTAAGTCCTCCGACACG	*C. pneumoniae*	This study
Cp740as	GAACAACGCCAACCATTACC	*C. pneumoniae*	This study
Cox748	GGTGGGTTATTTAACGGCG	*C. burnetii*	This study
Cox877	CACCTCCTTATTCCCACTCG	*C. burnetii*	This study
Mp2408s	AGGTGTTGGATTATGTGCCC	*M. pneumoniae*	This study
Mp2526as	CGTACTAATTCCGCTGGACG	*M. pneumoniae*	This study

2.2.4. Specific RT-PCR

Five specific RT-PCR procedures were applied to each DNA extract, using the primers shown in Table 1 and SYBR Green chemistry. *Bartonella* spp. was detected with primers targeting the citrate synthetase gene *gltA* (Norman et al., 1995). *Tropheryma whipplei* was identified with primers targeting specific regions (Fenollar et al., 2004). *Chlamydophila pneumoniae* was identified by amplifying part of the major outer membrane protein-encoding gene. *Coxiella burnetii* was detected with primers targeting the transposase gene. *Mycoplasma pneumoniae* was detected by amplifying part of the adhesin P1 gene.

2.2.5. Purification and sequencing of PCR products

Each amplification product obtained by 16S rDNA PCRs was purified with the MSB Spin PCRapace kit® (Invitek, Eurobio, Courtaboeuf, France) and sequenced on both strands by using the BigDye Terminator v. 1.1 Cycle Sequencing kit® (Applied Biosystems) and an ABI PRISM 3130*xl* sequencer® (Applied Biosystems). Sequences thus obtained were compared with those 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 on 97% (Drancourt et al., 2000).

2.3. Conventional microbiology data

For each patient, we collected the results of blood culture, HV culture, and serology.

2.3.1. Blood cultures

Sets of aerobic (BacT/ALERT SA) and anaerobic (BacT/ALERT FN) bottles had been sampled and incubated for 30 days at 37 °C using the BacT/ALERT 3D (bioMérieux, Craponne, France) automated blood culture system. Bacterial isolates had been identified with the Vitek2 system (bioMérieux), and antibiotic susceptibility had been determined with the disk diffusion method, as recommended by the French Antibiogram Committee (http://www.sfm-microbiologie.org).

2.3.2. HV Examination

For each patient, HV tissue had been inoculated on a chocolate agar plate supplemented with Polyvitex (Becton Dickinson, Diagnostics, Le Pont de Claix, France) for culture at 37 °C with 5% CO_2 for 2 days, and in aerobic and anaerobic bottles at 37 °C for 10 days. Gram staining had also been performed on the HV sample.

2.3.3. Serology

Serum samples had been tested for antibodies to *C. burnetii* (*C. burnetii*-Spot IF®, bioMérieux; IFA IgM Fièvre Q Focus® and IFA IgG Fièvre Q Focus®, Focus Diagnostics, Eurobio, Courtaboeuf, France); *M. pneumoniae* (Platelia *M. pneumoniae* IgM TMB® and Platelia *M. pneumoniae* IgG TMB®; Bio-Rad, Marnes-la-coquette, France); *Bartonella quintana* and *Bartonenlla henselae* (IFA IgM *Bartonella* Focus® and IFA IgG *Bartonella* Focus®; Focus Diagnostics), and *Chlamydophila* spp. (Sero FIA IgM *Chlamydia*®, and Sero FIA IgG *Chlamydia*®; Savyon Diagnostics, DMO, Marne-la-vallée, France).

3. Results

We studied HV samples from 31 patients. The results of Gram staining, culture, serologic tests, and PCR are listed in Tables 2 and 3.

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