TITLE: Infective endocarditis: does a new 16S rDNA set of primers improve the microbiological diagnosis?

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Abstract

Background: In infective endocarditis (IE), blood cultures are negative in 2.5–31% of cases because of an antimicrobial treatment previously prescribed. Molecular methods may represent an alternative to conventional microbiological techniques in order to identify the causative agent.

Objectives: The aim of this prospective study was to evaluate the performance of a new primer pair (341F/785R) for 16S rDNA amplification in heart valves compared with primers 91E/13BS already used for the diagnosis of IE. 341F/785R primer pair was previously selected *in silico* to allow 16S rDNA amplification for a large coverage of bacterial species.

Results: Seventy-four patients suspected of infective endocarditis (IE) were included in this study. Infective endocarditis was diagnosed in 55 of these patients using the modified Duke criteria, which was the gold standard here. 91E/13BS primers were more sensitive than 341F/785R primers: 38/55 (69.1%) samples were positive using 91E/13BS primers against 28/55 (50.9%) with 341F/785R ($p=0.013$). When at least one of the two molecular methods was positive, the sensitivity and specificity of 16S rDNA amplification was 72.7% and 94.7%, respectively.

Conclusion: Even if the new primer pair 341F/785R seemed promising *in silico*, it was less sensitive for 16S rDNA amplification in heart valves than the 91E/13BS pair already used. This study underlines a lack of standardisation for 16S rDNA amplification on clinical samples.

Introduction

Infective endocarditis (IE) remains a serious infectious disease: indeed, the incidence has not changed much in recent times and mortality is still high (ranging from 9.6 to 26%) [1]. A collaborative approach between infectious disease specialists, surgeons and microbiologists is required to treat these patients, and a conventional microbiological culture is essential to find the etiologic agent and to adapt the antimicrobial treatment. Among the modified Duke criteria validated in several studies to make a positive diagnosis of endocarditis, blood cultures (BCs) remain the gold standard to isolate and identify the etiologic agent [2, 3]. Unfortunately, BCs are negative in 2.5–31% of suspected IE cases with an average of 10–15% based on to three reasons: prior antibiotic treatment, fastidious organisms such as *Brucella* spp. or fungi, and intracellular bacteria such as *Coxiella burnetii*, *Bartonella* spp., or *Tropheryma whippelii* [4-8]. In the case of surgical IE, heart valve culture can be performed to identify the causative agent, especially when the BCs remain negative. Unfortunately, the same reasons previously cited may explain a negative culture result.

To overcome these problems, culture-independent molecular techniques based on 16S rDNA gene sequencing have been developed and applied to many samples (e.g. blood, cardiac valve). These polymerase chain reactions (PCRs) are suitable for laboratories able to develop and validate these new molecular techniques with dedicated personnel [9-15]. The efficiency of 16S rDNA PCR amplification is dependent on primer sequences. Ideally, there should be no primer-template mismatches. A number of nucleotide sequences of 16S rDNA are highly conserved, and they are therefore often chosen for primer design. However, even these conserved sequences still vary to some degree for different bacterial species. Recently, the bacterial coverage and phylum spectrum of 512 primer pairs were evaluated *in silico*, in order to reduce the bias in PCR-based microbial studies. One primer pair (341F/785R) seemed

particularly promising according to Klindworth et al. [16]. This primer pair showed excellent coverage of bacterial species, with few primer-template mismatches. However, this 341F/785R primer pair was only evaluated for PCR-based microbial studies and not for the identification of pathogenic bacteria directly from clinical samples.

The aim of the present work was to compare the sensitivity of 16S rDNA PCR amplification in heart valves using these new 341F/785R primers, versus the older ones (91E/13BS) [17] already utilized in the diagnosis of IE.

Materials and methods

Clinical Specimens and Patients

Consecutive native heart valve specimens sent to the Clinical Bacteriology Laboratory between January 1, 2014 and December 31, 2014 were included. Eighty-two heart valves and four vegetations were collected from 74 patients. These fragments were aseptically removed from patients submitted to cardiac surgery for suspected IE. They were sampled and transported in sterile containers. The heart valves were processed in class 2 biosafety cabinet. The clinical features (Duke criteria) and other biological parameters were prospectively collected as follows: blood cultures sampled before and during surgery, antimicrobial therapy prescribed, and serology for intracellular bacteria.

Conventional culture

The valve tissue was vortexed beforehand in 1 ml of brain-heart broth. It was then cultured onto 5% sheep blood, chocolate + isovitalex agar and in a paraffined brain-heart broth and a Todd-Hewitt broth. The plates were incubated both in an anaerobic atmosphere and under a carbon dioxide atmosphere (5%) for 10 days at 37°C. The broths were kept for 15 days. A

fragment of the valve was deep frozen at -80°C. The isolated bacteria were identified by MALDI-TOF mass spectrometry (Bruker Daltonics, Wissembourg, France). Results of the May-Grünwald-Giemsa and Gram stains were reported as direct microscopic examination of imprints as follows: rare (≤ 1 leukocyte or bacteria per oil immersion [X 1000] field), 1+ (2–9 per oil immersion [X 1000] field), 2+ (10–50 per oil immersion [X 1000] field), and 3+ (> 50 per oil immersion [X 1000] field).

Molecular Microbiology

A sample of the heart valve was excised and added to a bead-containing tube with 200 μ L of PCR-grade water for mechanical lysis with Magna Lyser (Roche Diagnostic, Mannheim, Germany). The NucleoSpin Tissue (Macherey-Nagel, Hoerd, France) was used following the manufacturer's instructions. The resulting 100 μ L of eluate was stored at -20°C until utilization. The following primers were used: PCR1 (old set): 91E (5'-TCAAAGGAATTGACGGGGGC-3') and 13BS (5'-GCCCCGGGAACGTATTCAC-3'); PCR2 (new set) 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3'). Using *Escherichia coli* K12 as a reference template (Genbank accession number NR_102804.1), 91E/13BS produces a 478-bp amplicon whereas 341F/785R produces a 465-bp amplicon.

PCR optimization of cycling conditions, primer concentration and $MgCl_2$ concentration was carried out using a *Staphylococcus aureus* DNA template and infected heart valve samples. The employment of a purified Taq polymerase was necessary for 16S rDNA sequencing from clinical samples because Taq polymerases are often contaminated with bacterial DNA [18]. The PCR amplification mixture (50 μ L) was composed of: 5 μ L of PCR Buffer 10X (Eurogentec, Seraing, Belgium), 3 μ L of $MgCl_2$ (25 mM), 2 μ L of dNTP (20 mM), 2.5 μ L of forward primer (10 μ M), 2.5 μ L of reverse primer (10 μ M), 0.25 μ L of purified Hot Diamond

Taq polymerase (5 U/ μ L) (Eurogentec, Seraing, Belgium), 29.75 μ L of PCR-grade water, and 5 μ L of extracted DNA. The following PCR conditions were used: for 91E/13BS - initial denaturation at 95°C for 3min, then 30 cycles of 94°C for 30s, 53°C for 30s, 72°C for 45s, and a final extension step at 72°C for 7 min; for 341F/785R - initial denaturation at 95°C for 3min, then 33 cycles of 94°C for 30s, 55°C for 30s, 72°C for 45s, and a final extension step at 72°C for 7min. Amplification of albumin was used as a positive extraction control.

Each amplification product was purified using Nucleospin Gel PCR Clean-Up (Macherey Nagel). Big Dye Terminator v3.1 kit was utilized for DNA sequencing. The same primers pairs were employed for sequencing. PCR conditions were 25 cycles of 94°C for 10s, 50°C for 5s, and 60°C for 240s. PCR products were purified onto NucleoSeq columns (Macherey-Nagel, Hoerd, France). DNA was sequenced using a 3500 Dx Genetic Analyzer (Life Technologies, Cergy-Pontoise, France). Species identification was based on Genbank sequences yielding a $\geq$ 99% sequence similarity score, and genus identification on Genbank sequences yielding a $\geq$ 97% sequence similarity score [19].

Results

A total of 74 patients (54 men, 20 female, median age = 61) were included in this study corresponding to 39 mitral valves, 39 aortic valves, 3 tricuspid valves, 1 pulmonary valve, and 4 vegetations. IE was diagnosed for 55 of these patients using modified Duke criteria. Conventional culture identified a microbiological agent for 48/55 patients (87.2%): 43 (78.2%) for blood culture and 16 (29.1%) for heart valve culture (Table 1). An antimicrobial treatment that could hinder the valve culture was initiated before surgery in 50/55 cases (90.9%). Bacteria were seen on the Gram stain for 14/55 patients (25.5%) (Table I and II).

16S rDNA could be amplified with at least one set of primers in 40/55 cases (72.7%). 91E/13BS primers were more sensitive than 341F/785R primers: 38/55 (69.1%) samples were positive using 91E/13BS primers against 28/55 (50.9%) with 341F/785R (Table 2). This difference was statistically significant using the McNemar test ($p=0.013$). 16S rDNA amplification was positive in all patients with bacteria present on the Gram stain (14/14, 100%), whereas it was positive in only 26/41 (63.4%) patients where no bacteria had been seen on the Gram stain.

For 19 patients, the diagnosis of IE was excluded using the modified Duke criteria, and they were included as controls. *Streptococcus mitis/oralis* DNA was amplified using 16 rDNA sequencing in 1 of these cases (5.3%). This patient was hospitalized for a recent valvular regurgitation and presented a 38.5°C fever, but blood cultures remained sterile. Even if the patient had not fulfill Duke's criteria, IE could not be ruled out as *viridans*-group streptococci are often associated with endocarditis.

Seven patients had culture-negative endocarditis. 16S rDNA was amplified for 5/7 (71%) of these patients. The empirical antimicrobial therapy was ineffective for patient number 45 (Table 2): the patient was treated with third-generation cephalosporins but *Enterococcus faecalis* DNA was found in his aortic heart valve using 16S rDNA PCR. On the other hand, the empirical antimicrobial therapy could be de-escalated for patients 13, 18 and 49. Patient 73 corresponded to a *Bartonella* endocarditis (Table III). Finally, no causative microbiological agent could be identified for 2 patients who had a definite diagnosis of IE. Finally, when at least one of the two molecular methods was positive, the sensitivity and specificity of 16S rDNA amplification was 72.7% and 94.7%, respectively.

Discussion

The aim of this study was to evaluate a new set of primers (341F/785R) showing an excellent bacterial coverage in the *in silico* evaluation performed by Klindworth *et al.* [16]. However, the sensitivity of this primer pair was lower than expected in the work presented here when working directly from heart valve samples. One possible explanation is that the 341F/785R primers are impacted by the background human DNA present in heart valves, reducing PCR efficiency. Indeed, the 341F DNA sequence contains a degeneracy of 8 (1 N and 1 W), and 785R a degeneracy of 9 (1 H and 1 V). A higher degeneracy increases bacterial coverage, but also increases non-specific amplification. The performance of PCR assays could also be affected by the length of the PCR products, but this length was similar for 91E/13BS and 341F/785R (478-bp and 465-bp, respectively). The 341F/785R primer pair did not seem particularly adapted in this study with samples containing a high human DNA background. The classical set (91E/13BS) seems to be more sensitive in the case of IE, although the new set (341F/785R) allowed us to identify a microbiological agent in two cases not detected by the old primer set (patients 38 and 58).

The work presented here confirms the results of previous studies using conventional 16S rDNA PCR on large cohorts of valve samples (51 to 245) showing a sensitivity and specificity ranging from 41.2 to 67% and from 91 to 100%, respectively, whereas the sensitivity and specificity of valve cultures ranged from 7.8 to 23% and 93.3 to 100%, respectively [9, 11-13, 15, 20]. Recently, real-time PCR applied to 177 and 357 valve samples [13, 14] showed sensitivities ranging from 80.6-96% and specificities ranging from 95.3-100%, respectively, whereas the sensitivities and specificities of valve culture were 13-33.4% and 96.6-100%, respectively.

The 16S rDNA PCR successfully detected bacteria even after effective antimicrobial therapy. The detection of bacterial DNA in cardiac valves does not mean that an active valve infection

is evolutive in so far as 16S rDNA PCR cannot differentiate viable from non-viable microorganisms. On the contrary, the results may remain negative if the inoculum is too low, and do not exclude the IE diagnosis based on Duke's criteria (2 cases in this study). In the case of IE, even when BCs were positive, molecular techniques are advised to consolidate the existing diagnosis [21]. Various authors have proposed the addition of the molecular techniques results into the Duke classification as a major criterion [22]. Indeed, as previously described by Marin [13] and by Vondracek [15], molecular techniques identify a causal agent in 4/53 (8.5%) and 3/35 (7.5%) of culture-negative IE cases, respectively. In the prospective study described here, a causative agent was identified only by 16S rDNA PCR in 5/55 (9%) of culture-negative IE cases.

Here, the new primer pair 341F/785R was less sensitive for 16S rDNA amplification on heart valve samples than the older 91E/13BS primer pair. The selection of appropriate primer pairs for 16S rDNA amplification on clinical samples seems critical, and this study underscores a lack of standardization for this molecular method. Conventional microbiological culture (BC and valve culture) remains the gold standard to document the etiology of IE. 16S rDNA amplification may be proposed in second-line when the culture of the valve remains sterile.

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276 **Table I:** Conventional microbiological and molecular 16S rDNA results for 55 patients with definite
277 IE

P	Valve	Gram stain*	Blood culture	Valve Culture	91E/13BS
1	Aortic + Tricuspid	N	<i>S. aureus</i>	S	<i>S. aureus</i>
2	Aortic	N	<i>Streptococcus sp.</i>	S	Abs
3	Mitral	N	<i>S. anginosus</i>	S	<i>S. anginosus</i>
4	Aortic	N	<i>S. sanguinis</i>	S	<i>S. sanguinis</i>
7	Mitral	N	<i>S. mitis / oralis</i>	S	<i>S. mitis/oralis</i>
8	Mitral	++	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
9	Mitral + Vegetation	N	<i>S. aureus</i>	S	<i>S. aureus</i>
10	Aortic	N	<i>S. aureus</i>	S	Abs
11	Aortic + Mitral	+++	<i>S. bovis</i>	S	<i>S. gallolyticus</i>
12	Aortic	+++	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
13	Aortic + Pulmonary	++	N	S	<i>S. oralis / pneumo</i>
14	Mitral + Vegetation	+++	<i>S. aureus</i>	<i>S. aureus</i>	<i>S. aureus</i>
16	Mitral	N	<i>S. aureus</i>	S	Abs
17	Aortic + Mitral	N	<i>S. aureus</i>	S	<i>S. aureus</i>
18	Pulmonary	N	N	S	<i>S. epidermidis</i>
19	Aortic	+++	<i>Serratia sp.</i>	S	<i>Serratia sp.</i>
20	Aortic	N	<i>S. pneumoniae</i>	S	<i>S. oralis / pneumo</i>
21	Aortic + Vegetation	+++	<i>S. gordonii</i>	<i>S. gordonii</i>	<i>S. gordonii</i>
24°	Mitral	N	N	S	Abs
25	Aortic + Mitral	N	<i>S. gallolyticus</i>	S	<i>S. gallolyticus</i>
29	Mitral + Vegetation	+	<i>S. dysgalactiae equisimilis</i>	S	<i>S. dysgalactiae equ</i>
30	Aortic	N	<i>G. adiacens</i>	S	<i>Granulicatella sp.</i>
34	Mitral	N	<i>S. epidermidis</i>	S	<i>S. epidermidis</i>
35	Aortic	+++	<i>S. oralis</i>	S	<i>S. oralis</i>
36	Aortic	N	<i>E. faecalis</i>	S	Abs
38	Aortic + Mitral	++	<i>S. gallolyticus</i>	S	Abs
42	Aortic	N	<i>S. aureus</i>	S	Abs
43	Aortic + Mitral	+++	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
44	Aortic	N	N	<i>S. gallolyticus</i>	<i>S. gallolyticus</i>
45	Aortic	N	N	S	<i>E. faecalis</i>
46	Mitral	+++	N	<i>C. hominis</i>	<i>C. hominis</i>
47	Aortic	N	<i>S. bovis</i>	S	<i>S. gallolyticus</i>
48	Mitral	N	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
49	Aortic	N	N	S	<i>S. gallolyticus</i>
50	Mitral	N	<i>S. mutans</i>	<i>S. mutans</i>	<i>S. mutans</i>
51	Aortic	N	<i>S. gallolyticus</i>	S	<i>Streptococcus sp.</i>
52	Aortic	N	<i>P. acnes</i>	S	Abs
53	Mitral	N	N	<i>P. avidum</i>	<i>P. avidum</i>
54	Aortic	N	<i>S. epidermidis</i>	S	<i>S. epidermidis</i>
55	Mitral	N	<i>S. sinensis</i>	S	Abs

56	Aortic + Tricuspid	N	<i>S. bovis</i>	S	Abs
57	Mitral	N	<i>S. aureus</i>	S	Abs
58	Aortic	N	<i>S. gallolyticus</i>	S	Abs
59	Aortic + Mitral	++	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>
60	Aortic	N	<i>E. faecalis</i>	S	<i>E. faecalis</i>
62	Mitral	N	<i>S. aureus</i>	<i>S. aureus</i>	<i>S. aureus</i>
63	Aortic + Mitral	N	<i>S. aureus</i>	<i>S. aureus</i>	<i>S. aureus</i>
64	Mitral	++	N	<i>S. mitis/oralis</i>	<i>S. mitis/oralis</i>
66	Aortic	N	<i>S. mitis/oralis</i>	S	<i>S. mitis/oralis</i>
67	Mitral	N	<i>S. aureus</i>	<i>S. aureus</i>	Abs
69	Mitral	N	N	S	Abs
71	Mitral	N	<i>E. faecalis</i>	S	Abs
72	Aortic	N	<i>C. albicans, E. faecalis, E. coli</i>	S	Abs
73	Tricuspid	N	N	S	<i>B. quintana</i>
74	Aortic	N	N	<i>G. adiacens</i>	Abs

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279 Abbreviations: P = patient ; N= negative ; S = sterile ; Abs = absence of amplification ; ATB =
280 Antimicrobial therapy before surgery

281 *Gram stain: 1+ (2–9 per oil immersion [X 1000] field), 2+ (10–50 per oil immersion [X 1000] field),
282 3+ (> 50 per oil immersion [X 1000] field)

283 ° Patient with a positive *Bartonella* serology

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285 **Table II:** Value of conventional and molecular (16S rDNA) techniques for the diagnosis of IE

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	Total number	Gram stain	Heart valve culture	Blood culture	16S rDNA PCR 91E	16S rDNA PCR 341F	16S rDNA PCR 91E or 341F
DEFINITE IE	55	14 (25.5%)	16 (29.1%)	43 (80.0%)	38 (69.1%)	28 (50.9%)	40 (72.7%)
Culture Positive	48 (87.3%)	13 (27.1%)	16 (33.3%)	43 (90.0%)	33 (68.8%)	25 (52.1%)	35 (72.9%)
Culture Negative	7 (12.7%)	1 (14.3%)	0	0	5 (71.4%)	3 (42.9%)	5 (71.4%)
ABSENCE of IE	19	0	0	2 (10.5%)	1 (5.3%)	0	1 (5.3%)

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289 **Table III:** Patients with culture negative endocarditis

Patient PCR		91E/13BS	341F/785R	Antibiotics	Comment
13	positive	<i>S. oralis/S. pneumoniae</i>	<i>S. oralis/S. pneumoniae</i>	Yes : AMC, GEN, VAN,TF	Wide spectrum antimicrobial therapy
18	positive	<i>S. epidermidis</i>	<i>S. epidermidis</i>	Yes : TZP, VAN, CAN	Wide spectrum antimicrobial therapy
45	positive	<i>E. faecalis</i>	<i>E. faecalis</i>	Yes : C3G	Ineffective antimicrobial therapy
49	positive	<i>S. gallolyticus</i>	<i>S. gallolyticus</i>	Yes : AMC, GEN	Adapted antimicrobial therapy
73	positive	<i>Bartonella quintana</i>	<i>Bartonella quintana</i>	Yes : AMC, GEN, DOXY	Positive <i>Bartonella</i> serology
24	negative			Yes : AMC	Positive <i>Bartonella</i> serology
69	negative			Yes : AMX	

290 Abbreviations : AMC = amoxicillin-clavulanic acid ; GEN = gentamicin ; TF = fluconazole ; VAN = vancomycin ; TZP = piperacillin tazobactam ; CAN =
 291 caspofungin ; C3G = third generation cephalosporin ; DOXY = doxycycline ; AMX = amoxicillin

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