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

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1 **TITLE:** Infective endocarditis: does a new 16S rDNA set of primers improve the
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23

24 **CONFLICT OF INTEREST:** None

25

26 **Abstract**

27
28 *Background:* In infective endocarditis (IE), blood cultures are negative in 2.5–31% of cases because
29 of an antimicrobial treatment previously prescribed. Molecular methods may represent an alternative
30 to conventional microbiological techniques in order to identify the causative agent.

31 *Objectives:* The aim of this prospective study was to evaluate the performance of a new primer pair
32 (341F/785R) for 16S rDNA amplification in heart valves compared with primers 91E/13BS already
33 used for the diagnosis of IE. 341F/785R primer pair was previously selected *in silico* to allow 16S
34 rDNA amplification for a large coverage of bacterial species.

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

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

44

45 **Introduction**

46

47 Infective endocarditis (IE) remains a serious infectious disease: indeed, the incidence has not
48 changed much in recent times and mortality is still high (ranging from 9.6 to 26%) [1]. A
49 collaborative approach between infectious disease specialists, surgeons and microbiologists is
50 required to treat these patients, and a conventional microbiological culture is essential to find
51 the etiologic agent and to adapt the antimicrobial treatment. Among the modified Duke
52 criteria validated in several studies to make a positive diagnosis of endocarditis, blood
53 cultures (BCs) remain the gold standard to isolate and identify the etiologic agent [2, 3].
54 Unfortunately, BCs are negative in 2.5–31% of suspected IE cases with an average of 10–
55 15% based on to three reasons: prior antibiotic treatment, fastidious organisms such as
56 *Brucella* spp. or fungi, and intracellular bacteria such as *Coxiella burnetii*, *Bartonella* spp., or
57 *Tropheryma whippelii* [4-8]. In the case of surgical IE, heart valve culture can be performed to
58 identify the causative agent, especially when the BCs remain negative. Unfortunately, the
59 same reasons previously cited may explain a negative culture result.

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

70 particularly promising according to Klindworth *et al.* [16]. This primer pair showed excellent
71 coverage of bacterial species, with few primer-template mismatches. However, this
72 341F/785R primer pair was only evaluated for PCR-based microbial studies and not for the
73 identification of pathogenic bacteria directly from clinical samples.

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

77

78 **Materials and methods**

79 Clinical Specimens and Patients

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

88 Conventional culture

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

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

99 Molecular Microbiology

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

110 PCR optimization of cycling conditions, primer concentration and MgCl₂ concentration was
111 carried out using a *Staphylococcus aureus* DNA template and infected heart valve samples.
112 The employment of a purified Taq polymerase was necessary for 16S rDNA sequencing from
113 clinical samples because Taq polymerases are often contaminated with bacterial DNA [18].
114 The PCR amplification mixture (50 μ L) was composed of: 5 μ L of PCR Buffer 10X
115 (Eurogentec, Seraing, Belgium), 3 μ L of MgCl₂ (25 mM), 2 μ L of dNTP (20 mM), 2.5 μ L of
116 forward primer (10 μ M), 2.5 μ L of reverse primer (10 μ M), 0.25 μ L of purified Hot Diamond

117 Taq polymerase (5 U/ μ L) (Eurogentec, Seraing, Belgium), 29.75 μ L of PCR-grade water,
118 and 5 μ L of extracted DNA. The following PCR conditions were used: for 91E/13BS - initial
119 denaturation at 95°C for 3min, then 30 cycles of 94°C for 30s, 53°C for 30s, 72°C for 45s,
120 and a final extension step at 72°C for 7 min; for 341F/785R - initial denaturation at 95°C for
121 3min, then 33 cycles of 94°C for 30s, 55°C for 30s, 72°C for 45s, and a final extension step at
122 72°C for 7min. Amplification of albumin was used as a positive extraction control.

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

131 **Results**

132 A total of 74 patients (54 men, 20 female, median age = 61) were included in this study
133 corresponding to 39 mitral valves, 39 aortic valves, 3 tricuspid valves, 1 pulmonary valve, and
134 4 vegetations. IE was diagnosed for 55 of these patients using modified Duke criteria.
135 Conventional culture identified a microbiological agent for 48/55 patients (87.2%): 43
136 (78.2%) for blood culture and 16 (29.1%) for heart valve culture (Table 1). An antimicrobial
137 treatment that could hinder the valve culture was initiated before surgery in 50/55 cases
138 (90.9%). Bacteria were seen on the Gram stain for 14/55 patients (25.5%) (Table I and II).

139 16S rDNA could be amplified with at least one set of primers in 40/55 cases (72.7%).
140 91E/13BS primers were more sensitive than 341F/785R primers: 38/55 (69.1%) samples were
141 positive using 91E/13BS primers against 28/55 (50.9%) with 341F/785R (Table 2). This
142 difference was statistically significant using the McNemar test ($p=0.013$). 16S rDNA
143 amplification was positive in all patients with bacteria present on the Gram stain (14/14,
144 100%), whereas it was positive in only 26/41 (63.4%) patients where no bacteria had been
145 seen on the Gram stain.

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

152 Seven patients had culture-negative endocarditis. 16S rDNA was amplified for 5/7 (71%) of
153 these patients. The empirical antimicrobial therapy was ineffective for patient number 45
154 (Table 2): the patient was treated with third-generation cephalosporins but *Enterococcus*
155 *faecalis* DNA was found in his aortic heart valve using 16S rDNA PCR. On the other hand,
156 the empirical antimicrobial therapy could be de-escalated for patients 13, 18 and 49. Patient
157 73 corresponded to a *Bartonella* endocarditis (Table III). Finally, no causative
158 microbiological agent could be identified for 2 patients who had a definite diagnosis of IE.
159 Finally, when at least one of the two molecular methods was positive, the sensitivity and
160 specificity of 16S rDNA amplification was 72.7% and 94.7%, respectively.

161 **Discussion**

162 The aim of this study was to evaluate a new set of primers (341F/785R) showing an excellent
163 bacterial coverage in the *in silico* evaluation performed by Klindworth *et al.* [16]. However,
164 the sensitivity of this primer pair was lower than expected in the work presented here when
165 working directly from heart valve samples. One possible explanation is that the 341F/785R
166 primers are impacted by the background human DNA present in heart valves, reducing PCR
167 efficiency. Indeed, the 341F DNA sequence contains a degeneracy of 8 (1 N and 1 W), and
168 785R a degeneracy of 9 (1 H and 1 V). A higher degeneracy increases bacterial coverage, but
169 also increases non-specific amplification. The performance of PCR assays could also be
170 affected by the length of the PCR products, but this length was similar for 91E/13BS and
171 341F/785R (478-bp and 465-bp, respectively). The 341F/785R primer pair did not seem
172 particularly adapted in this study with samples containing a high human DNA background.
173 The classical set (91E/13BS) seems to be more sensitive in the case of IE, although the new
174 set (341F/785R) allowed us to identify a microbiological agent in two cases not detected by
175 the old primer set (patients 38 and 58).

176 The work presented here confirms the results of previous studies using conventional 16S
177 rDNA PCR on large cohorts of valve samples (51 to 245) showing a sensitivity and
178 specificity ranging from 41.2 to 67% and from 91 to 100%, respectively, whereas the
179 sensitivity and specificity of valve cultures ranged from 7.8 to 23% and 93.3 to 100%,
180 respectively [9, 11-13, 15, 20]. Recently, real-time PCR applied to 177 and 357 valve samples
181 [13, 14] showed sensitivities ranging from 80.6-96% and specificities ranging from 95.3-
182 100%, respectively, whereas the sensitivities and specificities of valve culture were 13-33.4%
183 and 96.6-100%, respectively.

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

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

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

202

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275

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

278

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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