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FULL PAPER

Is the Unyvero i60 ITI multiplex PCR system a promising test in the diagnosis of infective endocarditis from heart valves?

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SUMMARY

The aim of this study was to evaluate the new commercial Unyvero i60 ITI multiplex PCR system (Curetis, Holzgerlingen, Germany) on native cardiac valves in comparison with made in-house 16S rRNA PCR amplification (91E/13BS primers) and conventional microbiological techniques.

Forty-four patients (30 men, 14 women) with suspected infective endocarditis (IE) were included in this evaluation corresponding to 30 aortic valves and 14 mitral valves. IE was definite for 40 patients using the modified Duke criteria.

16S rRNA PCR amplification was successful in 22 patients (55%). The Unyvero i60 ITI cartridge yielded a positive result in 16 patients (40%). Among the 40 cases, the etiological agent was not included in the panel of Unyvero i60 ITI cartridge for 14 cases. Moreover, for *S. aureus*, the Unyvero i60 ITI cartridge quickly yielded the susceptibility to meticillin. The result of the experiment was available after 5 hours whereas 16S rRNA PCR amplification-sequencing needs 14 hours of manipulation.

If the manufacturer incorporates new targets able to detect more endocarditis agents such as viridans streptococci, the Unyvero i60 ITI cartridge may be a promising and easy-to-use test.

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INTRODUCTION

Infective endocarditis (IE) remains a serious infectious disease whose incidence has not changed in recent years and the mortality is high at around 20% (Habib *et al*., 2009). A collaborative approach involving the infectious disease specialist, surgeon and microbiologist is required to treat these patients and 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 (Durack *et al*., 1994), blood cultures (BCs) remain the gold standard to isolate and identify the etiologic agent (Heiro *et al*., 1998; Murdoch *et al.,* 2009). Unfortunately, samples are negative in 2.5 to 31% of suspected IE cases with an average of 10-15% for three reasons: prior antibiotic treatment, intracellular bacteria such as *Coxiella burnetii, Bartonella* spp. *Tropheryma whipplei*, and fastidious organisms such as *Brucella* spp. or fungi (Hoen *et al*., 1995; Berbari *et al*., 1997; Houpikian *et al*., 2005; Werner *et al*., 2008; Fournier *et al*., 2010). To overcome these problems, molecular techniques have been used in the last twenty years to find the etiologic agent of IE. However, if these culture-independent molecular techniques based on 16S rRNA gene sequencing have been developed and applied to many samples (e.g. blood, cardiac valve), they are made in-house techniques needing a strict quality validation and technical skills to obtain accurate results. Thus, these PCRs are suitable in laboratories able to develop and validate these new molecular techniques with dedicated personnel (Breitkopf *et al*, 2005; Goldenberger *et al*., 1997; Greub *et al*., 2005; Kotilainen *et al*. 2006; Marin *et al*., 2007; Vollmer *et al*., 2010; Vondracek *et al*., 2011). Recently, three studies using a commercial Septi*Fast* (SF) real-time PCR test (Roche Diagnostics, Mannheim, Germany) able to detect 25 microbial agents was evaluated on 15, 9 and 20 cardiac valves respectively, showing promising results (Fernandez *et al*., 2010; Mencacci *et al*., 2011; Leli *et al.*, 2014).

The aim of this study was to evaluate retrospectively the commercial Unyvero i60 ITI multiplex PCR system (Curetis, Holzgerlingen, Germany) on native cardiac valves in comparison with the made in-house 16S rRNA PCR amplification (91E/13BS primers) and conventional microbiological techniques (valve culture, blood culture). This new cartridge offers a broad panel of 114 genomic targets for identification of bacterial or fungal species typically recovered together with a set of antibiotic resistance markers (*Table 1*).

Key words:
Cardiac valve, Infective endocarditis, PCR, 16S rRNA amplification, Unyvero.

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MATERIAL AND METHODS

Clinical specimens and patients

Consecutive native heart valve specimens sent to the Clinical Bacteriology Laboratory between April 1, 2016 and October 31, 2016 were included. In all, 44 heart valves were collected from 44 patients. These fragments were asepti-

cally removed from patients submitted to cardiac surgery for suspected IE. They were sampled and transported in sterile containers. The heart valves were processed in a class 2 biosafety cabinet. The clinical features (Duke criteria) and other biological parameters were collected as follows: BCs sampled before and during surgery, antimicrobial therapy prescribed.

Table 1 - *Bacteria, fungi and resistance genes detected by the Unyvero system.*

Detected pathogens	*Antibiotic resistance markers*
Staphylococcus spp. *Staphylococcus aureus* *Staphylococcus epidermidis* *Staphylococcus haemolyticus* *Staphylococcus lugdunensis* *Staphylococcus saprophyticus* *Staphylococcus capitis* *Staphylococcus hominis*	*mec*A, *mec*C (LGA251) *aac*(6')/*aph*(2") *rpo*B (*S. aureus*) *erm*A, *erm*C *van*A, *van*B
Streptococcus spp. *Streptococcus agalactiae* *Streptococcus pyogenes* *Granulicatella adjacens* *Abiotrophia defectiva*	*aac*(6')/*aph*(2") *erm*A, *erm*C *van*A, *van*B
Enterococcus spp. *Enterococcus faecalis, Enterococcus faecium* *Enterococcus hirae* *Enterococcus sulfurens* *Enterococcus saccharolyticus* *Enterococcus mundii* *Enterococcus casseliflavus,* *Enterococcus gallinarum* *Enterococcus flavescens* *Enterococcus durans* *Enterococcus dispar* *Enterococcus columbae* *Enterococcus cecorum* *Enterococcus asini*	*erm*A, *erm*C *van*A, *van*B
Corynebacteriaceae *Corynebacterium genitalium* *Corynebacterium casei* *Corynebacterium striatum* *Corynebacterium jeikeium* *Corynebacterium aurimucosum* *Corynebacterium singularis* *Corynebacterium simulans* *Corynebacterium accolens* *Corynebacterium amycolatum* *Corynebacterium minutissimum* *Corynebacterium macginleyi* *Corynebacterium auriscanis*	
Enterobacteriaceae *Escherichia coli* *Enterobacter cloacae complex,* *Enterobacter aerogenes* *Proteus mirabilis, Proteus vulgaris,* *Proteus penneri Klebsiella oxytoca,* *Klebsiella pneumoniae*	*ctx*-M *vim* *imp* *kpc* *ndm* *oxa*-23, *oxa*-24/40, *oxa*-48, *oxa*-58
Non-fermenting bacteria *Pseudomonas aeruginosa,* *Acinetobacter baumannii complex*	*aac*A4 *gyr*A (*E. coli*)
Anaerobes *Propionibacterium acnes,* *Propionibacterium avidum/ granulosum* *Finegoldia magna* *Bacteroides fragilis group*	
Fungi *Candida parapsilosis* *Candida albicans*	

Conventional culture

The valve tissue was vortex mixed 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 (at x1000 magnification): rare (≤1 leukocyte or bacterium per oil immersion field), 1+ (2-9 per oil immersion field), 2+ (10-50 per oil immersion field), and 3+ (>50 per oil immersion field).

Molecular microbiology

In house 16S rRNA PCR method: A sample of the heart valve was excised and added to a bead-containing tube with 200 µl of PCR-grade water for mechanical lysis with Magna Lyser (Roche Diagnostic, Mannheim, Germany). The NucleoSpin Tissue (Macherey-Nagel, Hoerdt, France) was used following the manufacturer's instructions. The resulting 100 µl of eluate was stored at -20 °C until utilization. The following primers were used for the PCR 91E (5'-TCAAAKGAATTGACGGGGGC-3') and 13BS (5'-GCCCGGGAACGTATTCAC-3') (Gauduchon *et al*., 2003). Using *Escherichia coli* K12 as a reference template (Genbank accession no. NR_102804.1), 91E/13BS produced a 478 bp amplicon. PCR optimization of cycling conditions, primer concentration, and $MgCl_2$ concentration were carried out using a *Staphylococcus aureus* DNA template and heart valve samples. The PCR amplification mixture (50 µl) was composed of: 5 µl of PCR buffer 10 X (Eurogentec, Seraing, Belgium), 3 µl of $MgCl_2$ (25 mM), 2 µl of dNTP (20 mM), 2.5 µl of forward primer (10 µM), 2.5 µl of reverse primer (10 µM), 0.25 µl of purified Hot Diamond *Taq* polymerase (5 U/µl) (Eurogentec), 29.75 µl of PCR-grade water, and 5 µl of extracted DNA. The following PCR conditions were used: for 91E/13BS - initial denaturation at 95°C for 3 min, then 30 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. Amplification of albumin was used as a positive extraction control. Each amplification product was purified using Nucleospin Gel PCR Clean-Up (Macherey-Nagel, Hoerdt, France). Big Dye Terminator v3.1 kit was used for DNA sequencing. The same primer pairs were employed for sequencing. PCR conditions were 25 cycles of 94°C for 10 s, 50°C for 5 s, and 60°C for 240 s. PCR products were purified onto NucleoSeq columns (Macherey-Nagel, Hoerdt, France) after agar gel electrophoresis. DNA was sequenced using a 3500 Dx Genetic Analyzer (LifeTechnologies, Cergy-Pontoise, France). Species identification was based on Genbank sequences yielding a ≥99% sequence similarity score, and genus identification if yielding a ≥97% sequence similarity (Drancourt *et al*., 2000).

Unyvero test: The Unyvero platform is an easy and fast system for the diagnosis of severe infectious diseases. The platform consists of different core components: the Unyvero Lysator, responsible for sample preparation, the Unyvero

Analyzer, which processes the Unyvero Cartridges and the Unyvero Cockpit. The i60 ITI Unyvero cartridge was processed as recommended by the manufacturer as described by Hischebeth *et al*. (Hischebeth *et al*., 2016). Briefly, a fragment of the valve was transferred in a sample tube with 180 µl of lysis buffer. After a 30 minute automated treatment including mechanical, chemical and enzymatical steps, the sample was transferred to the Unyvero cartridge with the mix PCR solution. After 4.5 hours of processing, the result was obtained by analysis with the manufacturer's software (Unyvero operation software OS 3.0).

Specific PCR: In case of negative made in-house 16S rRNA PCR amplification and positive Unyvero method, a specific PCR of the species was done with *nuc*, *sod*A, *opr*L primers for *S. aureus*, *Streptococcus* sp. and *P. aeruginosa* respectively as described in the literature (Barbier, 2009; Poyart *et al*., 1998; Billard-Pomares *et al.*, 2011).

RESULTS

A total of 44 patients (30 men, 14 women) were included in this evaluation corresponding to 30 aortic valves and 14 mitral valves. IE was definite for 40 patients using the modified Duke criteria (*Table 2*). In these 40 cases of IE, the results of the direct microscopic examination were positive in 10/40 samples (25%). Although an antimicrobial treatment was initiated in 39 patients at the time of surgery, valve cultures were positive in 19/40 samples (47.5%) whereas the BCs sampled before or during the surgery were positive in 33 patients (82.5%).

16S rRNA PCR amplifications performed on 40 IE samples were successful in 22 patients (55%): 7 *Streptococcus gallolyticus*, 5 *Staphylococcus aureus*, 2 *Streptococcus agalactiae*, 1 *Enterococcus faecalis*, 1 *Enterococcus faecium*, 1 *Streptococcus pneumoniae*, 1 *Streptococcus equinus*, 1 *Streptococcus anginosus*, 1 *Streptococcus constellatus*, 1 *Staphylococcus epidermidis*, 1 *Actinotignum schaalii*. The Unyvero i60 ITI cartridge yielded a positive result in 16 patients (40%): 8 meticillin-susceptible *Staphylococcus aureus,* 1 meticillin-resistant *Staphylococcus aureus,* 3 *Enterococcus faecalis,* 1 *Enterococcus* sp, 1 *Streptococcus agalactiae*, 1 *Pseudomonas aeruginosa* and 1 *Streptococcus pneumoniae* (*S. pneumoniae* was detected by the Unyvero P55 Pneumonia cartridge, initially adapted to the respiratory pathogens). No *van*A and *van*B gene were detected in *Streptococcaceae* with the Unyvero i60 ITI cartridge corresponding to the microbial sensitivity test performed. 16S rRNA PCR amplification and the Unyvero cartridge were positive in 8/10 and 5/9 patients respectively when bacteria were present on the Gram stain whereas they were positive only in 15/30 patients and 11/30 patients respectively when no bacteria had been seen on the Gram stain.

The Unyvero i60 ITI cartridge was negative in 24 patients, but in 14 cases, these results may be considered uninterpretable (*Table 2*): 12 cases where it was impossible to detect the etiological agent because the Unyvero i60 ITI panel did not contain the implicated species (7 *Streptococcus gallolyticus*, 1 *Streptococcus equinus*, 1 *Streptococcus anginosus*, 1 *Streptococcus constellatus*, 1 *Streptococcus gordonii*, 1 *Actinotignum schaalii*) and 2 technical failures. The 16S rRNA PCR amplification was negative in 18 patients. The lack of detection by 16S rRNA PCR amplification of *S. aureus*, *P. aeruginosa* had been corrected by specific PCR in 6 cases (5 *S. aureus* = patients 5, 6, 9, 19, 38, 1 *P. aeruginosa* = patient 4). On the other hand, no *sod*A PCR detected *Streptococcus* sp. in a case of negative 16S rRNA PCR in a patient with positive *Streptococcus* BCs. Performances of Unyvero i60 ITI depended on the causal agent. Thus, the sensitivity rates of Unyvero i60 ITI for staphylococci, enterococci and streptococci were 64.3%, 66.7% and 13.4% respectively, whereas the specificity was 100% for all microorganisms. All in all, the sensitivity of Unyvero i60 ITI taking the modified Duke criteria as gold standard, all microorganisms together, was 40% (versus 47.5% for valve culture and 55% for 16S rRNA). The specificity was 100%, whatever method was used.

DISCUSSION

The aim of this retrospective pilot study was to evaluate a new commercial molecular kit in infective endocarditis. As recommended by Munoz *et al.* (Munoz *et al*., 2008), heart valve cultures were performed for patients with a clinical suspicion of IE. In our 44 patients, 4 were excluded because cardiac echography did not show vegetation and the BCs were sterile. For the 40 evaluable samples, the Unyvero i60 ITI cartridge was positive in 40% of cases. This sensitivity level might be considered weak but corresponds to the low values found in the literature (Breitkopf *et al*., 2005; Maneg *et al*., 2014). These results are similar to those reported in the literature using 16S rRNA PCR amplification ranging from 33% to 96% on cohort of valve samples (n = 51-200) (Breitkopf *et al*., 2005; Greub *et al.,* 2005; Kotilainen *et al*., 2006; Vondracek *et al*., 2011, Maneg *et al.,* 2016; Miller *et al*., 2016). In these studies, the wide range of sensitivity distribution may be explained in the made in-house PCR assays by the wide range of primers used in the nucleotide positions in the 16S rRNA gene. Whatever the molecular technique used, the results are better than those obtained by conventional culture since the level of positivity does not exceed 50% (Kotilainen *et al*., 2006; Vollmer *et al*., 2010; Miller *et al.*, 2016). The 10 negative results obtained for the bacteria included in the Unyvero panel may be explained by a low inoculum as the microscopic examination showed no bacteria for 9 of them.

In our study, 16S rRNA PCR amplification detected 50% of the etiological agent with a lack of 4 *S. aureus*, confirmed by a specific *nuc* PCR. This study shows that the 16S rRNA PCR amplification may fail especially when the direct microscopic examination stain is negative (6/40). Moreover, for *S. aureus*, the Unyvero i60 ITI cartridge is able to detect the *mec* gene quickly suggesting resistance to meticillin. The result of the experiment was available after 5 hours whereas 16S rRNA PCR amplification-sequencing needs 14 hours of manipulation. Therefore, the great advantage of Unyvero PCR is the speed of the technique. Another benefit of the Unyvero i60 ITI cartridge multiplex PCR system is that it additionally provides several gene-coded resistance markers, so that specific antibiotic treatment could take place even on the day of the surgical procedure. This new molecular technique may successfully detect bacteria after effective antibiotic treatment and may explain the positivity in healed patient samples knowing that antibiotics and tissue infiltration influence the retention of pathogens. Nevertheless, the detection of bacterial DNA in cardiac valves does not mean an active valve infection as so far molecular techniques cannot differentiate viable from non-viable microorganisms. In case of IE, even when BCs were positive, molecular techniques are advised to consolidate the existing diagnosis (Gould *et al*. 2012; Millar *et al*., 2016). More-

Table 2 - *Conventional microbiological and molecular results for 40 patients with definite infective endocarditis.*

Patient	Valve	Microscopic examination		Culture valve	BC/surgery	16S rRNA	Unyvero	Antibiotic before the surgery	Leukocytes ($x10^9$/L)	CRP (mg/L)	BC before surgery
		Bacteria	Leukocytes								
1	Aortic	Abs	++	*E. faecium*	*E. faecium*	*E. faecium*	*Enterococcus sp*	YES	3.23	54	*E. faecium*
2	Aortic	Abs	+	MSSA	MSSA	*S. aureus*	MSSA	YES	18.73	272	MSSA
3	Aortic	Abs	+	Sterile	Not done	*S. gallolyticus*	Negative	YES	16.45	20	Not done
4	Mitral	Abs	Abs	Sterile	*P. aeruginosa*	Negative	*P. aeruginosa*	YES	14.19	5	Not done
5	Aortic	Abs	+	Sterile	Sterile	Negative	MSSA	YES	8.39	27	MSSA
6	Ao	Abs	+	MSSA	Not done	Negative	Negative	YES	8.21	28	MSSA
7	Aortic	+ GPC	++	*S. pneumoniae*	*S. pneumoniae*	*S. pneumoniae*	*S. pneumoniae**	YES	23,18	160	*S. pneumoniae*
8	Aortic	Abs	+	*P. mirabilis*	Sterile	Negative	Negative	YES	6,7	15	Sterile
9	Mitral	Abs	+++	Sterile	Sterile	Negative	MSSA	YES	16,55	54	MSSA
10	Mitral	+++ GPC	+++	MSSA	MSSA	*S. aureus*	MSSA	YES	20,06	35	MSSA
11	Aortic	Abs	Abs	*E. faecalis*	Not done	Negative	*E. faecalis/ Enterococcus spp*	NO	6,08	16	*E.faecalis*
12	Aortic	+++ GPC	+	*S.haemolyticus*	Sterile	Negative	Negative	YES	10,59	Not done	Not done
13	Mitral	+ GPC	++	MRSA	MRSA	*S.aureus*	MRSA	YES	21,92	49	MRSA
14	Aortic	Abs	Abs	Sterile	*S. equinus*	*S. equinus*	Negative	YES	4.62	ND	Not done
15	Mitral	Abs	+	MSSA	MSSA	*S. aureus*	MSSA	YES	21.15	229	Not done
16	Aortic	Abs	Abs	Sterile	*S.gallolyticus*	*S.gallolyticus*	Negative	YES	11,74	261	Not done
17	Aortic	+++ GPC	+++	*E.faecalis*	Not done	*E.faecalis*	*E. faecalis/ Enterococcus spp*	YES	8,7	84	*E.faecalis*
18	Mitral	Abs	+	Sterile	MSSA	Negative	MSSA	YES	5,11	31	Not done
19	Aortic	Abs	++	Sterile	Sterile	Negative	Negative	YES	8,89	30	MSSA
20	Aortic	Abs	+	Sterile	Sterile	*S. anginosus*	Negative	YES	7,28	8	*S. anginosus*
21	Mitral	Abs	++	*E. faecalis*	Not done	Negative	Negative	YES	5,68	6	Not done
22	Mitral	Abs	Abs	Sterile	*S.gordonii*	Negative	Negative	YES	14,73	6	Not done
23	Aortic	Abs	+	Sterile	*E.faecalis*	Negative	*E. faecalis/ Enterococcus spp*	YES	15,29	7	*E.faecalis*
24	Aortic	++ GPC	+	Sterile	Not done	*S.agalactiae*	*S.agalactiae*	YES	23,03	51	*S.agalactiae*
25	Aortic	Abs	+	Sterile	Sterile	*S.gallolyticus*	Negative	YES	8,35	59	*S.gallolyticus*
26	Aortic	Abs	Abs	*S.epidermidis*	Sterile	*S.epidermidis*	Negative	YES	8,39	15	*S.epidermidis*
27	Aortic	Abs	Abs	*A.schaalii*	*A.schaalii*	*A.schaalii*	Negative	YES	14,29	69	*A.schaalii*
28	Mitral	Abs	+	Sterile	Sterile	*S.gallolyticus*	Negative	YES	6,86	64	*S.gallolyticus*
29	Aortic	Abs	+	Sterile	Sterile	*S.gallolyticus*	Negative	YES	8,27	35	*S.bovis*
30	Aortic	Abs	Abs	Sterile	Sterile	Negative	Negative	YES	6,34	<3	Not done
31	Mitral	Abs	+++	MSSA	MSSA	*S.aureus*	MSSA	YES	24.12	113	MSSA
32	Mitral	Abs	++	Stérile	Sterile	Negative	Negative	YES	9,11	56	MSSA
33	Aortic	+++ GPC	++	*S.agalactiae*	Sterile	*S.agalactiae*	Invalid	YES	11,57	110	*S.agalactiae*
34	Aortic	+ GPC	+	*S.constellatus*	Sterile	*S.constellatus*	Negative	YES	36,23	55	Not done
35	Aortic	Abs	Abs	Sterile	*S.gallolyticus*	*S.gallolyticus*	Negative	YES	7,35	72	*S.gallolyticus*
36	Aortic	Abs	+	Sterile	Not done	Negative	Negative	YES	10,1	80	*S.agalactiae*
37	Aortic	+++ GPC	++	Stérile	Sterile	*S.gallolyticus*	Negative	YES	4,59	94	*S.gallolyticus*
38	Aortic	Abs	+++	MSSA	Sterile	Negative	MSSA	YES	9,59	61	MSSA
39	Mitral	++ YEASTS	++	*C.albicans*	Sterile	Negative	Invalid	YES	6,52	55	Not done
40	Mitral	Abs	Abs	Sterile	Sterile	Negative	Negative	YES	7,84	26	*E. faecalis*

Abs: absence; GPC: gram-positive cocci; MSSA: meticillin-susceptible *S.aureus*; MRSA: meticillin-resistant *S.aureus*.
*S. pneumoniae was detected by the Unyvero P55 Pneumonia cartridge, initially adapted to the respiratory pathogen.

over, this new molecular cartridge is easy to use and may be suitable in all laboratories without dedicated personnel. Indeed, the team of Malandain *et al*. recently used the new cartridge in the diagnosis of prosthetic joint infections with similar results (58% of concordance rate of Unyvero with culture) (Malandain *et al.*, 2018). The progress made by the manufacturer by incorporating new targets able to detect endocarditis agents such as *S. gallolyticus,* other viridans streptococci and a 16S rRNA primers is very promising to increase the level of detection of this new product. This new cartridge would yield better results than those obtained in the study of Leli *et al*. (Leli *et al*., 2014) using the SF kit with a 95% susceptibility from 20 cardiac valves knowing that this kit requires more hands-on technician time in the laboratory. Another alternative would be to use the blood cartridge (not available at the beginning of the study) to increase the sensitivity of the test because it includes a higher number of pathogens responsible for IE. The main drawback of this new technique is the cost of the instrument remaining high and representing a disadvantage for routine conventional testing in clinical laboratories. Consequently, this technology might be reserved to selected serious patients with infections such as infective endocarditis, severe sepsis, osteo-articular infections, and infection in immunocompromised patients. It is obvious that specific PCRs (with made in-house methods or commercial kits) are more efficient than 16S universal rRNA PCR. That is why Unyvero i60 ITI cartridge is a promising and easy-to-use test, especially after inclusion of new targets able to detect more endocarditis agents such as viridans streptococci.

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