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Proof of Concept Evaluation of a New Diagnostic POCT Device for Culture-Independent Microbiological Diagnosis of Infective Endocarditis

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Abstract

Introduction: Accurate and fast microbiological diagnosis of infective endocarditis (IE) is of vital importance for patient outcome.

Material & Methods: Forty culture-negative heart valves were evaluated with a new POCT multiplex-PCR cartridge (Unyvero™, Curetis AG, Holzgerlingen, Germany), advertised to detect several Gram positive/negative bacteria and fungi, together with several antibiotic resistance genes. Those POCT results were compared to conventional 16S rDNA PCR/sequencing results.

Results: POCT multiplex-PCR was positive in 13 cases [Staphylococcus aureus (n=5), Enterococcus spp. / *E. faecalis* (n=5), ConS (n=1), *Granulicatella adjacens* (n=1), *Abiotrophia adjacens* (n=1)]. Antibiotic resistances were found in 44 specimens, from which 2 specimens were without any pathogen identification. 16S rDNA PCR was positive in 20 cases. Consecutive sequencing identified those as *Staphylococcus spp.* (n=6), Enterococcus faecalis (n=4), *Streptococcus spp.* (n=4), *Leifsonia shinshuensis* (n=1), *Granulicatella elegans / G. adjacens* (n=2), *Abiotrophia adjacens* (n=1). One case was positive in 16S PCR without any reliable signal in sequencing. When comparing both methods, identification was consistent in 9 cases and divergent in other 9 cases.

Discussion: This POCT cartridge is easy to integrate into the daily microbiology laboratory work flow, and is less laborious than 16S sequencing PCR. For the application in routine IE diagnosis, the system needs to be optimized to include targets for viridans *streptococci* and HACEK group. In addition, problems with invalid resistance and pathogen target detection need to be fixed by the producer.

Conclusion: The analyzed POCT system might be a future diagnostic tool for IE detection following assay optimization.

Keywords: POCT; Microbiological diagnosis; Endocarditis; Antibiotic resistance

Introduction

Infective endocarditis (IE) is a serious disease with a high mortality rate [1] and accurate and fast microbiological diagnosis of IE is of vital importance for patient outcome. IE is difficult to diagnose as blood cultures and culture from heart valve tissue samples often remain negative, mainly due to previous microbial treatment or fastidious IE causing microorganisms (e.g. *Bartonella sp., Coxiella burnetti, Tropheryma whipplei*). For the culture identification of IE-causing HACEK (*Aggregatibacter aphrophilus, Cardiobacterium hominis, Eikenella corrodens, Kingella kingae*) bacteria and *streptococci*, a prolonged incubation for 7-14 days is often needed for identification [2]. Although the modified Duke criteria (DC) have been established for the diagnosis of IE, they have limitations in the diagnosis of IE in prosthetic valves and intra cardiac devices [3]. The yearly rate of IE in patients with a prosthetic valve is approximately 3 cases per 1,000 patients [4]. PCR may offer significant advantages in diagnosis due to the generation of fast results [5]. The correct identification of IE-causing pathogens is important for immediate targeted antibiotic therapy and the avoidance of unnecessary antibiotics, in the context of antibiotic stewardship (ABS) programs. Overall, there is a high need for optimized and faster molecular detection assays for early and targeted treatment of IE-causing pathogens [6-8].

Materials and Methods

POCT multiplex-PCR assay and conventional diagnosis

We performed an evaluation that examined the sensitivity and specificity of a new point of care testing (POCT) multiplex PCR assay (Unyvero™, Curetis, Holzgerlingen, Germany) for swift detection of causative microbes in culture-negative cases of IE. The Unyvero™ i60 ITI cartridge is advertised to detect several Grampositive/-negative bacteria and fungi in implant and tissue, together with some of the most important antibiotic resistance genes. Specimens from 40 fresh frozen heart valves were cultured on Columbia/chocolate agar plates and incubated in brain-heart broth for 7 days. In cases of no bacterial growth after 24 h, tissue specimens were evaluated with the new POCT multiplex-PCR assay for the detection of heart valve infections and compared to 16S rDNA PCR results. In cases of pathogen growth after >24 hours, identification of colonies was performed using MALDI-TOF (VITEK MS) or VITEK 2 technology. Heart valves were frozen after culture inoculation and thawed after 24 h for lysis in the POCT lysator which is able to process a wide range of clinical sample types using a standardized protocol. The Unyvero™ sample tube prepares the patient sample containing glass beads and buffers for bacterial lysis and sample liquefaction. The Unyvero™ i60 ITI cartridge is equipped with integrated reagent containers, a DNA purification column, eight separate PCR chambers and a corresponding number of arrays. The cartridge contains buffers for DNA purification, reagents and fluorescence-marked primers for PCR amplification, as well as probes for array hybridization and is assembled by inserting the Unyvero™ sample tube with the lysed sample and the master mix tube. Once assembled, the cartridge is physically closed, minimizing the risk of contamination. An internal control is also included in the cartridge in order to verify the DNA purification, PCR and array hybridization for each measurement. This gene is amplified in each of the eight PCR chambers and hybridized on each array The Unyvero™ analyzer processes up to two Unyvero™ Cartridges in random access mode and automatically performs DNA purification, specific amplification and detection. The Unyvero™ cockpit is equipped with a touchscreen and connects the Unyvero™ analyzer to the lysator. Resistance genes that can be measured included mec A/C (resistance to methicillin, and other ß-lactams), van A/B (resistance to glycopeptides), erm A/C (erythromycin-resistance genes), vim/imp/kpc/ndm (carbapenemases, metallo-β-lactamases), aacA4 (aminoglycoside 6'-N-acetyltransferase, resistance-modifying enzyme gene), ctx-M (most prevalent extended-spectrum beta-lactamases), rpoB oxa-23/-24/-48/-58(carbapenemases), gyrA (quinolone-resistance), aac(6‘)/aph(2‘‘) (aminoglycoside-6’-N-acetyltransferase/2’’-O-phosphoryltransferase).

16S PCR/sequencing

Bacterial DNA was automatically extracted by the Maxwell Tissue DNA Purification Kit on a Maxwell 15 machine (Promega, Mannheim, Germany). Amplification was performed using the Multiplex PCR Kit (Qiagen, Hilden, Germany) and the ligonucleotides 16S-F (5’ TGGTAGTCCACGCCGTAACC 3’) and 16S-GRP-R (5’ TCATAAGGGGCATGATGAT 3’) to detect Gram positive pathogens or 16S-GRN-R (5’ CGTAAGGGCCATGATGACT 3’) to detect Gram negative pathogens, respectively. The amplification was carried out on a Master cycler epi gradient S (Eppendorf, Hamburg, Germany) with the following conditions: initial denaturation at 95°C for 15 min, 35 cycles of 94°C for 15 sec and 53°C for 15 sec and 72°C for 1 min, followed by terminal elongation at 72°C for 5 min. The quality of each PCR run was ensured by negative control, positive control (DNA of *M.luteus* for Gram-positive PCR and DNA of *M.catarrhalis* for Gram-negative PCR) and inhibition control.

All PCR amplicons obtained from patient samples were subject to sequencing in both sense and antisense directions performed with a Big Dye Terminator v1.1 cycle sequencing kit and an ABI Prism 310 sequencer (Applied Bio systems, Foster City, CA, USA) according to standard protocols. Sequencing was performed by using forward and reverse primers (10 pmol each). PCR amplicons were purified from the gel with an Invisorb spin DNA extraction kit (Invitek, Berlin, Germany), and further treated with the sequencing kit material. The following program was executed: 1 cycle at 96°C for 1 min and 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The electropherog rams obtained were analyzed using sequencing analysis software (version 3.7; Applied Biosystems). The results were aligned and examined by Gen Bank NCBI genetic sequence database searching.

Results

Fresh frozen heart valves were examined in routine bacteriology laboratory and with the Unyvero™ i60 ITI Cartridge.

The ITI cartridge signal was positive in 13 cases [Staphylococcus aureus (n=5), *Enterococcus spp. / E. faecalis* (n=5), ConS (n=1), *Granulicatella adjacens* (n=1), *Abiotrophia adjacens* (n=1)] and negative in 27 cases. Problems with invalid targets measuring the full resistance panel occurred in 11 out of 40 cartridges. Antibiotic resistances were found in 4 specimens [1: vanB, rpoB, oxa-58, ndm; 2: ermC, oxa-48, aacvA4; 3: rpoB; 4: aac(6)/aph(2)). From these, two specimens (1;2) were without any pathogen identification. The detected pathogen related to the detected rpoB resistance was *S. aureus* and the detected species related to the detected aac(6)/aph(2) resistance was *E. faecalis.* 16S rDNA PCR was negative in 20 cases, and positive in 20 cases (**Table 1**). All 20 amplificates were sequenced, 19 of them were identified as *Staphylococcus spp* (n=6), *Enterococcus faecalis* (n=4), *Streptococcus spp.* (n=4), *Leifsonia shinshuensis* (n=1), *Granulicatella elegans / G. adjacens* (n=2), *Abiotrophia adjacens* (n=1). One case was positive in 16S PCR without signal in sequencing. When comparing 16S PCR results to Unyvero results, identification was consistent in 9 cases (4 x for *E. spp./ E.faecalis,* 3 x for *S. aureus,* 1 x for *Granulicatella adjacens,* 1 x for *Abiotrophia adjacens*) and divergent in 9 cases (2 x *Streptococcus spp.* (Seq.) versus 2 x *S. aureus* (ITI), 2 x *Streptococcus spp.* (Seq.) versus negative result (ITI), 1 x *Leifsonia* (seq.) vs. negative result (ITI); 3 x *S. aureus* (seq). vs. negative (ITI); 1 x *Granulicatella adjacens* (seq) vs. negative result (ITI). Bacteria from the HACEK group and *Streptocci* (with the exception of group A and B *streptococci*) are not covered in

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the ITI panel. Sensitivity/specificity analysis was not applied due to the small amount of specimen tested.

Table 1. Microorganisms and resistance markers detected by the Unyvero ITI Panel.

Group	Pathogen
Gram-positive bacteria	*Staphylococcus aureus*
	Coagulase negative *staphylococci* [15]
	Streptococcus agalactiae
	Streptococcus pyogenes [14]
	Enterococcus faecalis
Nutritionally variant streptococci	*Granulicatella adiacens*
	Abiotrophia defectiva
Corynebacteriaceae	*Corynebacterium spp [16]*
Enterobacteriaceae	Escherichia coli
	Enterobacter cloacae complex
	Enterobacter aerogenes
	Proteus spp. [1]
	Klebsiella pneumonia [17]
	Klebsiella oxytoca
Nonfermenters	*Pseudomonas aeruginosa*
	Acinetobacter baumannii complex
Anaerobic bacteria	*Propionibacterium acnes*
	Propionibacterium avidum+/granulosum[*]
	Finegoldia magna
	Bacteroides fragilis group [6]
Fungi	*Candida parapsilosis*
	Candida albicans
marker	Resistance
mecA	Oxacillin/methicillin
mecC (LGA251)	Oxacillin/methicillin
aac(6')/aph(2")	Aminoglycosides
ermA	Macrolides/lincosamides
ermC	Macrolides/lincosamides
vanA	Vancomycin
vanB	Vancomycin
rpoB	Rifampin
ctx-M	3 rd generation cephalosporins
Vim	Carbapenem
Imp	Carbapenem
Kpc	Carbapenem

Ndm	Carbapenem
aacA4	Aminoglycosides
gyrA (Escherichia coli)	Fluoroquinolones
oxa-23	Carbapenem
oxa-24	Carbapenem
oxa-48	Carbapenem
oxa-58	Carbapenem

Discussion

PCR testing of explanted heart valves is recommended in addition to culture techniques to increase diagnostic yield [9]. In case of negative culture result, current guidelines recommend that tissues from excised heart valves or vegetations from patients with suspected IE should be referred for broad-range bacterial PCR and sequencing [10]. Unfortunately, this 16S PCR is prone to contamination of reagents with bacterial DNA, which represents a major problem exacerbated by the highly sensitive nature of 16S PCR. These methods are also laborious and time-consuming, thus fully-automated assays which can be easily integrated in the routine work flow are needed. This evaluation highlights the importance of molecular analysis in diagnostically challenging culture-negative IE as time to result is of critical importance in the diagnosis of IE. For the majority of pathogens detected in this study, the information provided by the new POCT seems to be sufficient in the first place, given the high incidence of *staphylococci, streptococci and enterococci* in this disease. Although bacteria from the HACEK group were not discovered with 16S PCR IE, they should be covered by a POCT system, despite their rareness (**Table 2**).

Table 2. comparison results between conventional bacteriology, 16S PCR/sequencing and POCT.

No	Conventional bacteriology	16S PCR /sequencing	POCT result	POCT resistence genes
1	*E. faecalis*	positive /*Enterococcus spp.*	*Enterococcus spp.*	-
2	no growth	16S	PCR: negative	-
3	no growth	16S	PCR: negative	vanB, rpoB, oxa-58, ndm
4	no growth	16S	PCR: negative	-
5	no growth	16S	PCR: negative	-
6	no growth	16S	PCR: negative	ermC, oxa-48, aacvA4
7	no growth	16S	PCR: negative	-
8	no growth	16S	PCR: negative	-
9	*E. faecalis*	positive / *E. faecalis*		-
10	no growth	positive / *Streptococcus spp.*	*S.aureus*	-
11	*S. aureus*	positive / *Streptococcus spp.*	*S. aureus*	
12	no growth	positive / *S. spp. (S. aureus/S. haemolyticus*	negative	-
13	no growth	16S PCR: negative	negative	-
14	no growth	positive / *Leifsonia shinshuensis*	negative	-
15	no growth	positive / *S. aureus/ S. haemolyticus*	*S. aureus*	-
16	no growth	positive / *S. aureus/ S. haemolyticus)*	*S. aureus*	-
17	no growth	positive / *S. sanguinis*	negative	-
18	no growth	positive / *S. aureus*	*S. aureus*	rpoB
19	no growth	positive / *G. elegans*	*Enterococcus spp.*	-
20	no growth	positive / *A. adjacens*	*A. defectiva*	-
21	no growth	positive / *Staphylococcus spp.*	negative (2x)	-

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22	no growth	16S PCR: negative	negative	-
23	C. striatum P. acnes	16S PCR: negative	negative	-
24			CoNS	-
25	no growth	positive / *G. adjacens*	*G. adjacens*	-
26	no growth	positive / *S. sanguinis*/*mitis*	negative	-
27	E. faecalis	positive / *Enterococcus spp.*	*E. faecalis* *E. spp.*	aac(6)/aph(2)
28	E. faecalis	positive / *Enterococcus spp*	*E. faecalis* *E. spp.*	
29	no growth	positive	negative	-
30	no growth	16S PCR: negative	negative	
31	no growth	16S PCR: negative	negative	-
32	no growth	positive / *Staphylococcus spp.*	negative	-
33	no growth	16S PCR: negative	negative	-
34	no growth	16S PCR: negative	negative	-
35	no growth	16S PCR: negative	negative	-
36	no growth	16S PCR: negative	negative	-
37	no growth	16S PCR: negative	negative	-
38	no growth	16S PCR: negative	negative	-
39	no growth	16S PCR: negative	negative	-
40	no growth	16S PCR: negative	negative	-

The detection of 5 *staphylococcal* cases using multiplex PCR assay is not surprising, as *Staphylococcus aureus* is described as the most common cause of IE in the developed world [11]. There was only a slight difference in the absolute numbers of *staphylococcal* cases using 16S rDNA PCR/sequencing (6 cases, although *Staphylococcus spp* in 6 cases. *Streptococcal species* are common causative IE pathogens [7]. This is in line with our finding of 4 *streptococcal* cases in this evaluation. Accurate identification within some *streptococcal* groups was limited with both techniques used. In case of divergent results, such results should be interpreted with caution. Biochemical streptococcal species identification were shown to result in false identifications in more of half of the patients when compared to genetic discrimination methods (**Table 3**). Nutritionally variant streptococci (*Abiotrophia defective or Granulicatella spp.),* are thought to account for 2% of all infective endocarditis cases [12]. Due to difficulties in obtaining positive microbiology cultures, *Granulicatella adjacens* is known to be responsible for culture-negative infective endocarditis [13]. Both methods used in this evaluation were convergent for *Abiotrophia spp,* and one *Granulicatella* case, but divergent for 2 other cases of *Granulicatella*.

Thus these two results should be interpreted with caution. Infections caused by multi-resistant enterococci (*E. faecium, E. faecalis*) have increased over the recent years to a point that they now represent the 3rd most common cause of IE worldwide [14].

Table 3. Overall Comparison of POCT results to Standard conventional 16S PCR/Sequencing results.

Results	**N**
Convergent negative with both methods	20
Convergent positive with both methods	9
16S PCR positive / Unyvero negative	6
16S PCR negative / Unyvero positive	1
divergent positive results	3
true negative	21
true positive	18
false negative	6
false positive	4

This is also reflected in our evaluation. Unlike *streptococci* and *taphylococci*, most enterococci do not produce a set of potent pro-inflammatory toxins, but they are equipped with many genes encoding adhesion proteins that may mediate adherence to host tissues, consistent with their pathogenic role in infective

endocarditis. *Vancomycin-resistnat E. faecium* (VRE) is difficult to treat [15].

Conclusion

The Unyvero ITI cartridge could represent a useful tool for IE diagnosis. It can be easily integrated into the lab work flow and is less laborious. However, for its application in routine IE diagnosis the multiplex system needs to be optimized and extended to include targets for viridans *streptococci* and the HACEK group. Therefore, a new specific IE cartridge needs to be developed. In addition, problems with invalid resistance and pathogen target detection need to be fixed before routine testing.

Acknowledgement

Preliminary data of this work were demonstrated in part as poster presentation at the 2015 Annual Meeting of the German Society of Hygiene and Microbiology (Deutsche Gesellschaft für Hygiene und Mikrobiologie; DGHM) in Münster, Germany. This evaluation was performed at the Institute of Medical Microbiology, Jena University Hospital, and supported by Curetis AG, the manufacturer of the molecular diagnostic prototype tool. POCT system and PCR cartridges were provided for free over a test period of six months.

Conflict of Interest (COI) Statement

All authors report no COI

Ethical standards statement

This evaluation has been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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