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Evaluation of Commercial Universal rRNA Gene PCR plus Sequencing Tests for Identification of Bacteria and Fungi Associated with Infectious Endocarditis▽

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Two new commercially available universal rRNA gene PCR plus sequencing tests, SepsiTest and universal microbe detection (UMD; Molzym, Bremen, Germany), were evaluated using blood specimens and heart valves from 30 patients with suspected infectious endocarditis (IE). The sensitivity of PCR (85%) was nearly twice as high as that of culture (45%), which in 10/20 IE cases presumably stayed negative as a consequence of growth inhibition of the pathogens by antibiotics. Further, PCR provided the basis for reclassification of 5/10 non-IE cases into IE cases. Culture-negative infections were identified by PCR, including single infections due to streptococci and Gram-negative bacteria (*Escherichia coli*, *Haemophilus parainfluenzae*) and mixed infections involving two Gram-positive bacteria or *Candida* spp. with Gram-positive bacteria. The new commercial tests proved to be of value for the rapid diagnosis of IE, particularly in cases of culture-negative infections. Issues regarding the feasibility of these tests for routine use are discussed.

The incidence and relatively high mortality of infectious endocarditis (IE) has remained relatively constant over the past 30 years (14). Currently, the incidence of IE is approximately 3 to 10 episodes/100,000 person-years (6). The patient's outcome is dependent on various factors, including the specific infectious microorganism involved. The diagnosis of IE relies on clinical review, echocardiographical analysis, and blood culture diagnosis (6). Blood cultures are reported to stay negative in 2.5 to 31% of cases because of the inability of fastidious microorganisms to grow (6) and growth inhibition of pathogens due to prior antibiotic administration (9, 13). Such blood culture-negative infections (BCNIs) delay both the diagnosis and the initiation of adequate treatment, thereby profoundly impacting clinical outcome.

PCR methods followed by DNA sequence analysis are highly sensitive and specific tools for detecting and identifying the etiological agents of IE (14). Consistently high diagnostic sensitivity has been reported (12, 14, 21, 22). On the other hand, PCR is considered to be prone to false-positive results due to contamination of DNA extraction and PCR reagents (2) and also due to false-negative results as a result of PCR inhibitors coeluting with the DNA (8). In recent years, PCR tests that partially address these concerns have been commercially marketed for the detection and identification of common etiological agents of sepsis (3, 20, 23). These systems use extracted total DNA (SeptiFast; Roche), microbial DNA enriched by affinity chromatography (VYOO; SIRS-Lab), or selective lysis of blood cells (SepsiTest; Molzym). SeptiFast relies on real-time PCR, and VYOO relies on PCR analysis for the detection and identification of a limited number of microorganisms. SepsiTest employs real-time PCR plus sequence analysis of amplicons to detect and identify a broad range of pathogens. Because of the wide variety of etiological agents in IE, we evaluated SepsiTest for the analysis of whole-blood (WB) and heart valve (HV) samples from patients who were operated on for endocarditis.

MATERIALS AND METHODS

From July 2009 to April 2010, 30 patients who underwent surgery for endocarditis at our tertiary care hospital were classified according to the modified Duke criteria for the diagnosis of infective endocarditis (11). The Duke classification of IE includes major and minor criteria (6). The major criteria include results from blood cultures positive for a typical IE pathogen (e.g., viridans group streptococci, HACEK group organisms (2a), *Staphylococcus aureus*, or *Coxiella burnetii*), blood cultures persistently positive in repeated blood draws, or the presence of other clinical evidence strongly suggesting IE (e.g., echocardiography or valve regurgitation). The minor criteria are predisposition, fever, vascular or immunological phenomena, or microbiological evidence for IE other than the major criteria. A diagnosis of IE is considered definitive in patients with 2 major, 1 major and 3 minor, or 5 minor criteria. Among the 20 patients with a definitive diagnosis of IE in the current study, 17 fulfilled two major criteria, and 3 patients fulfilled 1 major and 3 minor criteria. All patients received perioperative and first day postoperative cefuroxime treatment. Definitive IE and some non-IE patients were treated with antibiotics at least 1 day before and/or 6 to 8 weeks after the operation (Table 1). All data presented in this work, including molecular studies, represent results from routine analysis of valvular tissue and blood samples collected from patients with suspected endocarditis.

Heart valves (HVs) were prepared for microbiological and molecular analyses immediately after resection. For this purpose, the HVs were aseptically cut into two pieces of approximately the same size using a sterile scalpel. One part was transferred directly into Hemoline diphasic performance bottles (bioMérieux, Nürtingen, Germany) and cultured for up to 21 days in the case of negative cultures. Single whole-blood (WB) samples were collected shortly before surgery. A volume of 8 to 10 ml was added to each of the aerobic and anaerobic blood culture bottles (BD Bactec Plus; Heidelberg, Germany) and incubated for 7 days in the case of negative cultures. Identification and antimicrobial susceptibilities were performed according to standard operating procedures in our microbiological laboratory (Vitek 2; bioMérieux).

The remaining HV portion was cut into small pieces and extracted according

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TABLE 1. Results of culture and PCR based on Duke classification of patients

Patient no.	Valve[b]	Treatment[c]		Organism result[a]				
		Pre	Post	Valve culture	Blood culture	Other[d]	Valve PCR	Blood PCR
Proven IE patients								
2	M, native	Van, Rif, Gen	Van, Rif	Negative	Negative	Vegetation on valve	*Streptococcus gallolyticus*	*S. gallolyticus*
3	A and M, native	Gen, Pen	Van, Gen, Rif	Negative	Negative	CoNS from CVC; *S. sanguinis* (+1-3 days)	*S. gordonii*[e]	*S. gordonii*
7	A, native	Van, Rif, Gen	Van, Rif	Negative	*Staphylococcus epidermidis*	Valve abscess	Negative	Negative
11	A, native	Amp, Cep	Amp, Gen	Negative	Negative	*S. mitis* in BC (−21 days)	*Streptococcus dysgalactiae*	*S. dysgalactiae*
12	A, prosthetic	Van, Rif Amp	Van, Rif, Amp	*Enterococcus faecalis*	Negative	Calcified valve	*E. faecalis*	*E. faecalis*
13	A, native	Pip, Sul	Taz, Gen, Sul	*E. faecalis*	*E. faecalis*	*E. faecalis* from spleen swab (−1 days)	*E. faecalis*	*E. faecalis*
14	A, prosthetic	Van, Gen, Rif	Van, Gen, Rif	*Staphylococcus lentus*	ND	*S. epidermidis* from valve (same day) and pleura swab (−2 days); calcified valve	*S. epidermidis*	*S. epidermidis*
16	A, native	Flu	Van, Gen, Rif	Negative	Negative	NA	*S. epidermidis*	*S. epidermidis*
17	M, prosthetic	Taz, Gen, Rif	Taz, Gen, Rif	Negative	Negative	*E. faecalis* in BC (−18 days)¸ calcified valve	*S. gallolyticus*	*S. gallolyticus*
18	A, prosthetic	Van, Gen	Van, Gen, Vor	*Candida albicans*	Negative	*S. hominis* in BC (−5 days); *C. albicans* in BC (−30 days)	*S. gallolyticus*, *C. albicans*	*S. gallolyticus*
19	A and M, native	Amp, Gen, Pen	Van, Gen	*Corynebacterium tuberculostearicum*	Negative	*S. gordonii* in BC (−7 days)	*S. gordonii*	*S. gordonii*
20	A, prosthetic	Van, Gen, Rif	Van, Gen, Lin	*S. epidermidis*	Negative	*S. epidermidis* from intraoperative swab	Negative	Negative
21	M, prosthetic	Amp, Gen	Amp, Gen	ND	Negative	NA	*S. gordonii*	*E. faecalis*
22	A, native	Van, Gen	Van, Gen, Pen	Negative	Negative	NA	Negative	*S. gordonii*
23	A, prosthetic, M, native	Van	Van	*E. faecalis*	*E. faecalis*	*E. faecalis* from valve swab	*E. faecalis*	*E. faecalis*
24	A, prosthetic	Gen, Amp, Sul	Van, Gen, Amp	*E. faecalis*	Negative	*E. faecalis* in BC (−17 days)	*E. faecalis*	Negative
26	M, native	None	Van, Gen	ND	Negative	*Streptococcus uberis*, *E. faecalis* in BC (−39 days)	*E. faecalis*	Negative
27	T, native	Flu, Cli	Flu, Cli	Negative	Negative	*Bacillus subtilis* from drainage (−16 days), *S. aureus* from pacemaker swab (−61 days)	Negative	Negative
28	A, native	Flu, Cli	None	Negative	Negative	CoNS from CVC swab (−19 days), *C. glabrata* in BC (−10 days)	*S. epidermidis*, *C. glabrata*	Negative
30	A, native	Pip, Sul	Gen, Cep, Cip	Negative	Negative	*Haemophilus parainfluenzae* in BC (+6 days); calcified valve	*H. parainfluenzae*	Negative
Non-IE patients								
1	A, native	None	None	Negative	Negative	NA	Negative	Negative
4	M, native	None	None	Negative	Negative	Abscess and degenerate valve tissue	*S. gordonii*	Negative
5	A, native	None	Van, Gen	Negative	Negative	NA	Negative	Negative
6	M, native	Dap, Mer, Flc	None	Negative	Negative	*E. faecium* from decubitus wound swab	Negative	*E. faecium*
8	A, native	None	Cli	Negative	Negative	NA	Negative	Negative
9	A, native	None	None	Negative	Negative	NA	Negative	Negative
10	A and M, native	None	None	ND	ND	NA	*S. epidermidis*[e]	Negative
15	A, native	None	None	ND	Negative	NA	*S. epidermidis*	*S. epidermidis*
25	M, prosthetic	None	None	Negative	Negative	*E. coli* in BC (−5 days)	*S. epidermidis*, *S. mitis* group	*E. coli*
29	A, native	None	None	Negative	Negative	NA	Negative	*S. epidermidis*

[a] NA, not available; ND, not determined.
[b] A, aortic; M, mitral; T, tricuspid valve.
[c] Preoperative (Pre) and postoperative (Post) treatment. All patients received perioperative cefuroxime treatment (see Materials and Methods). Amp, ampicillin; Cep, ceftriaxone; Cip, ciprofloxacin; Cli, clindamycin; Dap, daptomycin; Flc, fluconazole; Flu, flucloxacillin; Gen, gentamicin; Lin, linezolid; Mer, meropenem; Pen, penicillin; Pip, piperacillin; Rif, rifampin; Sul, sultamicillin; Taz, tazobactam; Van, vancomycin; Vor, voriconazole.
[d] Relevant results from inspection, previous (−) or succeeding (+) blood cultures, or cultures from other material as indicated in relation to the time of blood/valve sampling. BC, blood culture; CoNS, coagulase-negative staphylococci; CVC, central venous catheter.
[e] Both aortic and mitral valves were positive.

to the protocol supplied by the manufacturer (UMD-Tissue; Molzym, Bremen, Germany). A WB sample (approximately 5 ml) from the same puncture for microbiological analysis was collected in an EDTA tube and processed in two 1-ml aliquots as instructed (SepsiTest; Molzym). Universal rRNA gene real-time PCR assays of bacterial and fungal DNA extracted from HVs and WB samples were performed in a DNA Engine Opticon system (Bio-Rad, Munich, Germany). All controls run with each series of experiments (internal, positive, and negative PCR controls) yielded the expected results. Molecular work was performed under UV-decontaminated laminar flow and PCR cabinets with standard precautions to avoid DNA contamination. Amplicons from positive reactions were sequenced and analyzed as described previously (23).

RESULTS AND DISCUSSION

Diagnostic values. A cohort of 24 male patients (mean age, 60 years; range, 37 to 78 years) and 6 female patients (mean age, 68 years; range, 51 to 84 years) was classified into 20 definitive IE and 10 non-IE patients. These patients underwent surgery, and their mortality rate was 10% (3/30 patients). Of the 3 patients who died, 1 was definitely diagnosed with IE, and IE was excluded in the other two patients. The study included 22 aortic, 11 mitral, and 1 tricuspid HVs. The PCR results from 34 HV and 30 WB samples (30 patients) were compared with culture results from 29 HV (26 patients) and 28 WB (28 patients) samples. The overall patient-based positivity (both IE and non-IE patients) from HV and WB analysis by PCR was 76.7% (23/30), compared to 34.6% (9/26) by the culture test. When the PCR and culture results were considered together, the positivity was 83.3% (25/30). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the PCR test were 85% (17/20), 40% (4/10), 73.9% (17/23), and 57.1% (4/7), respectively, and for the culture test, they were 45% (9/20), 100% (9/9), 100% (9/9), and 45% (9/20), respectively. Taking into account the results from both the PCR and culture methods, 85% (17/20) of the definitive IE patients were positive by HV analysis, and 95% (19/20; 14 WB samples) were positive by HV plus WB analysis. With a definitive IE-related positivity (HV and WB samples) of 85% (17/20), PCR testing contributed more to the diagnosis of IE than did culture testing (45%; 9/20).

False-negative results. Eleven patients (patients 2, 3, 11, 16, 17, 21, 22, 26 to 28, and 30) were culture negative (Table 1), of which all but one (patient 27) were PCR-positive (Table 1). Prior antimicrobial therapy probably contributed to the failure to recover organisms in culture that were detected by PCR, though it is important to recognize that the PCR procedure used in this study was designed to detect only viable microbes. This perspective is supported by conclusions from previously published studies of sepsis in which PCR-positive specimens remained culture negative, presumably due to prior antimicrobial therapy (17, 23). In some patients, only a single blood culture specimen was collected, so it is possible that inadequate sampling may have contributed to false-negative cultures, though patients with IE are generally persistently bacteremic (22). Among the 10 PCR-positive culture-negative cases, the results from 5 patients were consistent with additional microbiological findings, including previous (patients 11, 26, and 28) or subsequent (patients 3 and 30) blood, catheter, or swab cultures (Table 1). Another 3 cases had concordant HV and WB PCR results (patients 2, 16, and 17). With the remaining 2 patients, PCR testing identified etiological agents typical of IE (18), including *Enterococcus faecalis* in a WB sample (patient 21), *Streptococcus gordonii* in an HV sample (patient 21), and *S. gordonii* in a WB sample (patient 22). Patient 27 was negative by both culture and PCR. This patient had developed an *S. aureus* urosepsis and infection of a pacemaker 2 months before the operation. Later testing of this patient showed an insufficiency of the tricuspid valve by echocardiography and vegetation on the pacemaker electrode, thus fulfilling two main Duke criteria. A probable reason why the culture was negative may be the extensive antibiotic treatment of the patient. The PCR-negative result may indicate a very low cell number or even the absence of the pathogen on the HV and in the bloodstream and hence successful treatment at the time of the operation.

PCR results for 3 definitive IE patients were negative, two of whom (patients 7 and 20) were culture positive for *Staphylococcus epidermidis* and one of whom was also culture negative (patient 27) (see above). The positive culture results were supported by a wound swab culture (patient 20) and the finding of a valve abscess (patient 7) as indicated in the computer tomography, supporting the suspicion of an infection. These results confirmed that the PCR results were false negatives. PCR inhibition is unlikely because internal controls indicated full performance of the assay (not shown). Wellinghausen et al. (23) discussed PCR-negative, culture-positive cases of sepsis as the result of pathogen DNA amounts being below the limit of the PCR detection system. This may also hold true for the PCR-negative results obtained here.

False-positive results. Although cultures of HV and WB samples from non-IE patients were expectedly negative throughout, positive results were observed in 6/10 non-IE patients by PCR analysis (Table 1). Although considered false positive according to the Duke classification, there is evidence that most of the PCR results are indicative of true infections. First, the finding of a common strain colonizing both aortic and mitral valves (patient 10) or present in both the HV and WB samples (patient 15) supports the hypothesis of true infection. Second, the *Enterococcus faecium* bacteremia found by PCR (patient 6) was supported by a wound swab taken at the time of the operation. The culture may have been negative because of the preoperative antibiotic treatment. Third, the identification of mixed *S. epidermidis* and *Streptococcus mitis* group strains on the HV and *Escherichia coli* bacteremia by PCR (patient 25) is difficult to explain by contamination. This patient did not receive pre- or postoperative antibiotic treatment. However, the cultures may have remained negative because of the perioperative application of cefuroxime. It is noteworthy that a blood culture analyzed 5 days earlier was positive for *E. coli*, which supports the PCR finding. Finally, the clinical history of patient 4 describes a former macroscopically visible severe IE with an abscess and degenerate HV. The PCR finding of *S. gordonii*, which is among the most frequent causative agents of IE (18), on the HV indicates a persisting viable but nonculturable infection (10). It is worth noting that the molecular tests employed eliminate free pathogen DNA during the extraction of the samples and thus detect only the DNA of viable microorganisms (7). The remaining patient (patient 29) was PCR positive for *S. epidermidis* in the WB sample. There was no further evidence of infection from microbiological or other diagnostic procedures, thus suggesting contamination during the blood sampling for PCR analysis. Therefore, in

TABLE 2. Organisms found in positive patients

Microorganism	Patient no.	No. of positive patients[a]						
		Total PCR + culture	Valve PCR (n = 20)	Blood PCR (n = 17)	Total PCR (n = 25)	Valve culture (n = 8)	Blood culture (n = 3)	Total culture (n = 9)
Gram-positive species								
Streptococcus spp.[b]	2, 3, 4, 11, 17, 19, 21, 22	8	7 (6)	7 (7)	8	0	0	0
Enterococcus spp.[c]	6, 12, 13, 21, 23, 24, 26	7	5 (5)	5 (4)	7	4 (4)	2 (2)	4
Coagulase-negative staphylococci[d]	7, 10, 14, 15, 16, 20, 29	7	4 (2)	4 (2)	5	2 (2)	1 (1)	3
Corynebacterium tuberculostearicum	19	1	0	0	0	1 (1)	0	1
Gram-negative species								
Escherichia coli	25	1	0	1 (0)	1	0	0	0
Haemophilus parainfluenzae	30	1	1 (1)	0	1	0	0	0
Fungi								
Candida albicans	18	1	0	0	0	1 (1)	0	1
Mixed strains								
S. epidermidis + *S. mitis* group	25	1	1 (0)	0	1	0	0	0
C. albicans + *S. gallolyticus*	18	1	1 (1)	0	1	0	0	0
C. glabrata + *S. epidermidis*	28	1	1 (1)	0	1	0	0	0

[a] Numbers in parentheses indicate organisms associated with proven IE patients. n = 25 positive patients.
[b] *S. dysgalactiae* (1); *S. gallolyticus* (2); *S. gordonii* (5).
[c] *E. faecalis* (6); *E. faecium* (1).
[d] *S. epidermidis* (7); *S. lentus* (1).

addition to complementing culture testing in the identification of pathogens in definitive IE patients, PCR may also aid in the identification of latent infections in patients who fail to meet Duke criteria for a diagnosis of IE. It is worth noting that the two non-IE patients who had fatal outcomes (patients 4 and 25) were PCR positive (Table 1).

Etiology. PCR contributed greatly to the detection of Gram-positive bacteria (streptococci, coagulase-negative staphylococci, and enterococci), which constituted the most abundant organisms in our population (23/25 positive patients) (Table 2), consistent with earlier findings (5, 15, 16). *Corynebacterium tuberculostearicum* was cultured from a valve (patient 19) and may represent a contamination artifact. This is supported by the fact that the PCR results indicated an *S. gordonii* infection in both the valve and the bloodstream. Notably, streptococci, Gram-negative *E. coli* and *Haemophilus parainfluenzae* and the yeast *Candida glabrata* were identified only by PCR. The failure of some organisms to grow does not appear to be an uncommon observation inasmuch as *H. parainfluenzae* and yeasts are difficult to grow in culture (1, 4, 16). Mixed infections were also detected solely by PCR, with the rate (12%) of which corresponding to other studies employing PCR (20, 23).

Conclusions. By identifying BCNIs (including mixed infections), the commercial system tested here proved valuable in the detection and identification of the etiological agents of IE. The tests overcome two major concerns regarding molecular techniques, i.e., variable performance due to PCR inhibition and reagent-borne contamination (9). In fact, we observed neither PCR inhibition nor any false-positive results in either the extraction controls (blood samples from healthy individuals) or the PCR-negative controls that are routinely set up in parallel with the samples (not shown). The analysis was performed within approximately 4 h, including DNA extraction and PCR analysis. At this point, a result indicating the presence or absence of bacteria and/or fungi in the sample is obtained. Sequence analysis took approximately 4 more hours or was conducted overnight. Vandercam et al. (19) calculated a cost of 93 euros per sample for a homemade universal PCR and sequencing test. This cost is comparable to that of HV analysis, which costs approximately 75 euros per positive sample (including 60 euros for extraction and PCR analysis and approximately 15 euros for amplicon cleanup and sequencing). The present results suggest that PCR assays for both HV and WB analyses may be needed for ultimate molecular detection of pathogens. This would potentially increase the cost of the analysis. In view of the considerable hands-on time (approximately 3 to 4 h) from extraction to sequence results and the variable costs, this test may be used rather selectively on BCNI cases for which information on targeted antibiotic therapy is required. However, some positive cases in this evaluation were discordant between culture and PCR results, with the latter indicating the true infectious agent (patient 19 (Table 1) or mixed infections (patients 18 and 28) (Table 2 and the discussion above). Further studies are required to clinically validate the PCR tests with respect to a cost-benefit analysis.

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