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Improvement of Detection of Bacterial Pathogens in Normally Sterile Body Sites with a Focus on Orthopedic Samples by Use of a Commercial 16S rRNA Broad-Range PCR and Sequence Analysis

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A new commercially available universal 16S and 18S rRNA gene PCR test, which is followed by sequence analysis of amplicons (SepsiTest), was evaluated for rapid identification of pathogens in the diagnosis of bone and joint infections. Eighty-three orthopedic samples and 21 specimens from other normally sterile body sites collected from 84 patients were analyzed in parallel by culture and PCR for detection of bacteria and fungi. Compared to culture, the diagnostic sensitivity and specificity of PCR were 88.5% and 83.5%, respectively. The detection rate of PCR (34.6%) was higher than that of bacterial culture (25.0%) as a consequence of the presence of fastidious and noncultivable species in samples and antibiotic treatment of patients. Thirteen culture-negative infections were identified by PCR, and PCR was able to detect culture-proven polymicrobial infections. On the other hand, three samples were culture positive but PCR negative. SepsiTest was demonstrated to be a valuable supplemental tool in the rapid detection of bacteria, especially for fastidious and noncultivable organisms, allowing earlier initiation of pathogen-adapted therapy in patients with bone and joint infections.

Rapid detection of pathogens in clinical samples is an important issue for better patient outcomes. In the last decade, molecular methods for the identification of bloodstream pathogens became more important due to their rapidity, sensitivity, and reproducibility. These methods are an attractive alternative when conventional bacteriological techniques fail to identify microorganisms, particularly slow-growing, fastidious, or noncultivable organisms. Interfering factors such as antimicrobial therapy may cause false-negative culture results even in cases of infections due to easy-to-culture pathogens such as staphylococci and streptococci (6, 16).

Molecular techniques include pathogen-specific, multiplex, and broad-range assays. The clinical usefulness of pathogen-specific techniques is limited by the large number of pathogens potentially involved in different kinds of infections. Multiplex real-time PCR assays, on the other hand, facilitate the rapid identification of pathogens and are a promising approach for routine use. Several studies have shown the value of this diagnostic tool for rapid detection of bloodstream pathogens, particularly in pretreated patients (4, 5, 25). Also, for clinical specimens other than blood, these assays show a great potential for diagnosing bacterial infections (21). An obvious limitation is the inability to detect microorganisms that are not included in the targeted spectrum of the multiplex PCR.

The use of PCR targeting conserved regions of microbial genomes, in particular the 16S rRNA of bacteria and the 18S rRNA of fungi, is a broad-range approach when combined with sequence analysis. This method potentially allows the direct detection of any cultivable or noncultivable bacterial or fungal pathogen. Specific applications of broad-range 16S and 18S rRNA PCR in clinical diagnosis include sepsis, endocarditis, meningitis and other central nervous system infections, and bone and joint infections (16, 22).

The SepsiTest (Molzym, Bremen, Germany), a commercial assay based on this technique, has been evaluated for different kinds of clinical samples like whole blood and heart valves (14, 23). The aim of this study was to evaluate SepsiTest for samples from other normally sterile body sites, with a focus on orthopedic samples such as synovial fluid (SF) and joint tissues (JT).

MATERIALS AND METHODS

Study population. One hundred four clinical samples from 59 patients of the University Hospital Innsbruck ($n = 78$), from 14 patients of four smaller district hospitals ($n = 15$), and from 11 patients of general practitioners in the Tyrol ($n = 11$) were collected prospectively at the Division of Hygiene and Medical Microbiology, Innsbruck Medical University, between September 2010 and February 2011. The majority of samples were SF ($n = 47$) and JT ($n = 36$). Additionally, 21 nonorthopedic samples, i.e., cerebrospinal fluid (CSF) ($n = 8$), heart valves (HV) ($n = 6$), peritoneal fluid ($n = 3$), lymph node tissue ($n = 1$), lung tissue ($n = 1$), and tissue samples obtained at postmortem examination (brain and liver, $n = 1$ each), were investigated. Samples were aseptically divided into two fractions, one each for the SepsiTest assay and for culture. Various microbiological samples from other body sites were also collected for testing when clinically indicated. Clinical data and the antimicrobial therapy administered were recorded on the day of sampling.

The study was approved by the ethics committee of the Innsbruck Medical University (Nr. 290/4.7).

DNA isolation. All samples were prepared for molecular analysis within 48 h after the specimens were obtained from the patients. DNA was extracted with the UMD Universal assay kit according to the protocol supplied by the manufacturer (Molzym, Bremen, Germany), and eluates were stored at −20°C. To avoid contamination of the DNA samples, DNA isolation was prepared in a laminar flow cabinet decontaminated daily by UV radiation and strictly separated from PCR processing.

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PCR and sequence analysis. The broad-spectrum rRNA real-time PCR assay was performed in a Light Cycler 2.0 instrument (Roche Diagnostics, Vienna, Austria) according to the manufacturer's instructions (SepsiTest, Molzym, Bremen, Germany). The procedure, described elsewhere in detail (23), includes real-time PCR analysis using primers targeting conserved regions of the 16S and 18S rRNA genes of bacteria and fungi, respectively. Sequencing of 16S rRNA amplicons employs two primers respectively encompassing Gram-negative and Gram-positive bacteria and a separate primer for 18S rRNA amplicons.

Amplicons from positive PCRs were purified with ExoSap-it (Affymetrix, Cleveland, OH) and sequenced using sequencing primers supplied in the SepsiTest kit. Sequence analysis of all amplicons was accomplished with the 3500 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Pathogens were identified by using the SepsiTest-BLAST tool database (http://www.sepsitest-blast.net) and the online search BLAST tool (2). Analysis of mixed sequences was performed using the Web-based RipSeq software (13). Genus and species identification was presumed to be correct for clinical samples with sequence identities of ≥97% and ≥99%, respectively, to reference sequences of strains in the database.

Interpretation criteria for discrepant results. A microorganism detected by PCR only was defined as a "true pathogen" if (i) this pathogen was cultured from further specimens collected from the same infectious site during the same infectious episode and/or (ii) the species was specific for the patient's type of infection. The term "possible pathogen" was assigned if the pathogen detected by only one method had been reported as a causative agent in the literature. "Indeterminate" was assigned to microorganisms with positive PCR results meeting neither the true nor the possible pathogen definition. A pathogen identified only by culture was regarded as true, as culture was defined as the gold standard for this study.

Culture. The specimens were immediately cultured on 5% sheep blood Columbia agar (Heipha, Eppelheim, Germany), chocolate agar (Heipha, Eppelheim, Germany), and Schaedler's agar (Oxoid, Basingstoke, United Kingdom) and incubated at 37°C in parallel in aerobic and anaerobic atmospheres for 48 h. Additionally, specimens were inoculated into brain heart infusion broth (Mast Group, Merseyside, United Kingdom) and thioglycolate broth (Becton Dickinson, Heidelberg, Germany) and incubated at 37°C for 7 days. Isolated colonies were identified with standard microbiological procedures. In brief, isolates were first evaluated based on plate morphologies after overnight growth. Colonies consistent with staphylococci were confirmed with rapid catalase, and *Staphylococcus aureus* was distinguished from coagulase-negative staphylococci (CoNS) with the Slidex Staph Plus latex agglutination test (bioMérieux, Marcy l'Etoile, France) and coagulase testing. CoNS were identified to group level and not further differentiated. Beta-hemolytic streptococci were grouped with the Phadebact latex agglutination test (Bactus AB, Huddinge, Sweden). Other streptococci and Gram-negative and anaerobic bacteria were identified with different commercial identification systems, principally the API and Vitek2 systems (bioMérieux).

Statistical analysis. Calculation of significance for comparison of SepsiTest and culture for detection of pathogens was performed by using the McNemar's test. A P value of <0.05 (two-tailed) was considered significant.

RESULTS

Of the 104 samples, 26 samples from 18 patients were culture positive. PCR yielded positive results for 36 samples from 25 patients. Among 47 synovial fluid specimens, 3 (6.4%) were positive with culture and 9 (19.1%) were positive with PCR. Fifteen (41.7%) culture-positive and 13 (37.1%) PCR-positive results were detected within 36 joint tissues. Twenty-one nonorthopedic specimens showed 38.1% positive-culture findings and 66.7% PCR-positive signals. The detection rate of PCR (34.6%) was higher than that of culture (25.0%) ($P = 0.02$). Compared to culture, the diagnostic sensitivity and specificity of the PCR were 88.5% and 83.5%, respectively. Only bacterial species and no fungal organisms were detected.

Eleven different bacterial species were found by culture and 16 species by PCR. Sequence analysis was successful with species identification in 34 cases and in one case at the genus level only (clostridial bacterium). In one case, PCR could not differentiate between *Shigella sonnei* and *Escherichia fergusonii.* All PCR controls run with each series of experiments (internal, positive, and negative PCR controls) yielded the expected results.

Congruence of culture and PCR results. Seventy-nine samples yielded congruent results regarding culture and PCR. Fourteen samples from 12 patients showed identical positive results. The following organisms were detected: *Staphylococcus aureus* ($n = 7$), CoNS/*Staphylococcus epidermidis* ($n = 4$), *Streptococcus pneumoniae* ($n = 2$), and *Neisseria meningitidis* ($n = 1$). Sixty-five specimens from 57 patients showed negative results by both methods. Thus, the concordance of PCR and culture for both identical positive and negative samples was (14 + 65)/104, i.e., 76.0%.

PCR-positive, culture-negative results. In 13 specimens from 11 patients, a positive PCR result in culture-negative samples was found (Table 1). These samples were distributed among six SF, one JT, three HV, one peritoneal fluid specimen, and two specimens from brain and liver tissue. PCR sequences of 11 different bacterial species were found. In two patients the PCR findings were supported by the detection of the same pathogen in other specimens from the same infectious site. Four of these 11 patients had received adequate antibiotic therapy at the time of sampling, including all three patients in whom a "true" pathogen was detected by PCR only. Of the remaining seven patients, five had been untreated. For two patients, no information about antibiotic pretreatment was available.

PCR-negative, culture-positive results. Three orthopedic samples were positive by culture but negative by PCR (Table 1). Two of them contained CoNS, and one contained *Streptococcus mitis*. In one case the culture finding was supported by culture results yielding the same pathogen (CoNS) in other specimens from the same infectious site.

Discordances in species identification. In 9 samples from 4 patients, culture and PCR identified different organisms: one of these samples was matched at the genus level (*Streptococcus mitis* versus *Streptococcus milleri*). The other samples with discordant species identification were linked to polymicrobial infections (Table 1). An infection was presumed to be polymicrobial if two or more microorganisms were detected by PCR or by culture or in other samples collected from the same infectious site during the same infectious episode.

Within 5 tissue samples from patient ID 89 (a case with prosthetic joint infection), polymicrobial infection with *Enterococcus faecalis* and CoNS (*Staphylococcus epidermidis*) was found by PCR in 2 samples and by culture in 1 sample. Each method missed one of the pathogens: PCR failed to detect *Enterococcus faecalis* in 3 samples, whereas culture missed *Staphylococcus epidermidis* in 4 samples. Several other specimens from the same infectious site (joint tissue from the knee) showed growth of both organisms.

One of two samples of hip tissue from patient ID 99 (a case with septic arthritis) yielded *Streptococcus dysgalactiae* by PCR but CoNS by culture, though both of them were found in other specimens during the same infectious episode.

TABLE 1 Discrepant test results for culture and PCR[a]

Patient ID	Diagnosis	Specimen(s)	No. of samples	Pathogen detected	No. of culture-positive samples	No. of PCR-positive samples	Further specimen(s) positive by culture (time)[b]
Detection of a single "true" pathogen							
7	Endocarditis	Heart valve tissue	2	*Streptococcus agalactiae*	0	2	*S. agalactiae* in blood culture (d10−)
84	Meningococcal sepsis	Brain, liver tissue	2	*Neisseria meningitidis*	0	2	None
13	Infected prosthetic joint	Hip tissue	1	*Streptococcus mitis*	1	0	None
40	Infected prosthetic joint	Joint tissue (knee)	1	CoNS[c]	1	0	CoNS in knee tissue and swab (d0)
86	Infected prosthetic joint	Joint tissue (knee)	1	CoNS	1	0	None
78	Spondylodiscitis	Spine tissue	1	*Streptococcus mitis*	0	1	None
				Streptococcus milleri	1	0	
Detection of >1 "true" pathogen							
89	Infected prosthetic joint	Joint tissue (knee)	5	*Enterococcus faecalis*	5	2	*S. epidermidis*, *E. faecalis* in knee joint tissue (d2−, d0)
				CoNS/*Staphylococcus epidermidis*[d]	1	5	
81	Fournier's gangrene	Peritoneal fluid	2	*Streptococcus pyogenes*	1	2	*S. pyogenes*, *B. fragilis*, *E. coli* in peritoneal fluid and tissue from abdomen (d0)
				Bacteroides fragilis	2	1	
				Escherichia coli	1	0	
99	Septic arthritis	Hip tissue	2	*Streptococcus dysgalactiae*	0	2	*S. dysgalactiae* in hip swab (d0), hip tissue (d1−), CoNS in joint tissue and swab from hip (d0, d2+)
				CoNS	1	0	
Detection of a "possible" pathogen							
12	Arthritis	Synovial fluid (knee)	1	Clostridial bacterium	0	1	None
52	Infected prosthetic joint	Synovial fluid (knee)	1	*Granulicatella adiacens*	0	1	*S. epidermidis* in synovial tissue from knee (d0)
27	Infected prosthetic joint	Synovial fluid (hip)	1	*Finegoldia magna*	0	1	None
50	Infected prosthetic joint	Synovial fluid (hip)	1	*Tropheryma whipplei*	0	1	None
83	Peritonitis, adnexitis	Peritoneal fluid	1	*Ureaplasma urealyticum*	0	1	None
4	Endocarditis	Heart valve tissue	1	*Pseudomonas aeruginosa*	0	1	None
41	Arthritis	Synovial fluid (knee)	1	*Staphylococcus epidermidis*	0	1	None
Indeterminate results							
79	Arthritis	Synovial fluid (knee)	1	*Shigella sonnei*/*Escherichia fergusonii*	0	1	None

[a] Results are for 25 specimens from 17 patients. See Materials and Methods for definitions of true and possible pathogens and indeterminate results.
[b] Time point of pathogen detection. d, days; −, pathogen detection before sampling; +, pathogen detection after sampling.
[c] CoNS, coagulase-negative staphylococci.
[d] Differentiation to group level by culture and to species level by PCR.

Both patients (ID 89 and 99) were under adequate antibiotic therapy at the time of sampling.

One of two peritoneal fluid samples of patient ID 81 yielded *Escherichia coli*, *Streptococcus pyogenes*, and *Bacteroides fragilis* by culture, whereas PCR missed *E. coli* but identified the other two pathogens. In the second sample, only one of three species was detected by each method: *Bacteroides fragilis* by culture and *Streptococcus pyogenes* by PCR (Table 1).

DISCUSSION

In this study, we analyzed 83 orthopedic samples and 21 specimens from other normally sterile body sites with SepsiTest, a new commercial PCR test. The concordance of positive and negative PCR and culture results was 76.0%. The sensitivity of PCR compared to culture was 88.5%, which is in keeping with the data from other studies that applied SepsiTest to analyze clinical samples: Wellinghausen and coauthors compared SepsiTest to blood culture for the diagnosis of bloodstream infections and found a sensitivity of 87% among 342 blood samples (23). Kühn et al. evaluated SepsiTest for patients with infectious endocarditis: 34 HV were investigated, and the sensitivity compared to culture was 85% (14).

Regarding PCR-positive but culture-negative results, we used predefined criteria to categorize the detected pathogen as a true or possible cause. Of 13 pathogens detected by PCR only, five were considered true and seven possible (one PCR result was indeterminate). "True" was assigned to three samples from two patients (IDs 7 and 99 [Table 1]) upon further, corroborating culture results, and to two samples from patient ID 84 due to PCR detection of a diagnosis-specific pathogen, i.e., *Neisseria meningitidis*, in autopsy samples from a case of meningitis. All three patients had received adequate antibiotic therapy, which may explain the negative culture results.

For the seven cases with possible pathogens, case reports underline the plausibility of the PCR result, i.e., *Pseudomonas aeruginosa* from an HV sample in endocarditis (1, 7, 17), *Ureaplasma urealyticum* in a female patient with peritonitis and adnexitis (3, 24), and *Staphylococcus epidermidis* from SF in an arthritis patient (9). The four further orthopedic samples yielded anaerobic, fastidious, or noncultivable organisms (i.e., a clostridial bacterium, *Finegoldia magna*, *Granulicatella adiacens*, and *Tropheryma whipplei*), each of which is a documented possible agent of arthritis (8, 10–12, 15, 18–20).

In the "indeterminate" case, PCR could not differentiate between *Shigella sonnei* and *Escherichia fergusonii*, and no cultural or clinical data supported the PCR result.

For three samples (2.9%) with PCR-negative but culture-positive results, PCR was considered false negative. False-negative PCR results have also been observed in the other studies evaluating SepsiTest: seven samples (2.0%) among 342 blood culture specimens (23) and two of 34 (5.9%) tested HV samples (14). An explanation for the negative PCR results could be the processing for the samples: they are split into two parts, and thus the lesser sample volume used for PCR analysis could result in a pathogen DNA amount being below the detection limit (23). In addition, the sensitivity of 1 CFU per specimen (as for well culturable pathogens) is difficult to attain by PCR.

It has been claimed that PCR assays cannot be used to identify each pathogen in cases of mixed infection (26). Nevertheless, our data demonstrate the simultaneous detection of two species by PCR in two of the three culture-proven cases of polymicrobial infection (Table 1). Although the combinations of all test results per patient give clear pictures, this is not the case at the level of the single specimen, as only 3 of 7 PCRs and 2 of 7 cultures yielded both pathogens. Therefore, our study supports the notion that a combination of methods is optimal for the detection of mixed infections. Kühn and coworkers found three cases of polymicrobial infection among 34 HV samples by SepsiTest only (14). In 11 of 342 blood samples, polymicrobial infection was detected by PCR only but was missed by PCR in four cases (23).

In conclusion, SepsiTest appears to be a valuable tool for diagnosing bone and joint infections in particular. In our opinion, the major advantage of the assay is its ability to detect and identify virtually any cultivable or noncultivable bacterial species and nonviable bacteria from patients treated with antibiotics. Nevertheless, SepsiTest should always be used in combination with culture, as this increases sensitivity, especially in cases of polymicrobial infections and when culture is indispensable for antimicrobial susceptibility testing. Further studies are needed to evaluate the cost-effectiveness of SepsiTest and, in the long run, the impact of this assay on the clinical outcome of patients.

REFERENCES

1. **Aggarwal A, et al.** 2011. Recurrent Pseudomonas aortic root abscess complicating mitral valve endocarditis. Heart Lung [Epub ahead of print.] doi:10.1016/j.hrtlng.2011.01.008.
2. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
3. **Bailey EA, et al.** 2002. Ureaplasma urealyticum CAPD peritonitis following insertion of an intrauterine device: diagnosis by eubacterial polymerase chain reaction. Perit. Dial. Int. **22**:422–424.
4. **Bloos F, et al.** 2010. A multicenter trial to compare blood culture with polymerase chain reaction in severe human sepsis. Intensive Care Med. **36**:241–247.
5. **Bravo D, et al.** 2011. Diagnostic accuracy and potential clinical value of the LightCycler SeptiFast assay in the management of bloodstream infections occurring in neutropenic and critically ill patients. Int. J. Infect. Dis. **15**:326–331.
6. **Breitkopf C, Hammel D, Scheld HH, Peters G, Becker K.** 2005. Impact of a molecular approach to improve the microbiological diagnosis of infective heart valve endocarditis. Circulation **111**:1415–1421.
7. **Dawson NL, et al.** 2011. Left-sided Pseudomonas aeruginosa endocarditis in patients without injection drug use. Medicine (Baltimore) **90**:250–255.
8. **Fenollar F, Levy PY, Raoult D.** 2008. Usefulness of broad-range PCR for the diagnosis of osteoarticular infections. Curr. Opin. Rheumatol. **20**:463–470.
9. **Fenollar F, Roux V, Stein A, Drancourt M, Raoult D.** 2006. Analysis of 525 samples to determine the usefulness of PCR amplification and sequencing of the 16S rRNA gene for diagnosis of bone and joint infections. J. Clin. Microbiol. **44**:1018–1028.
10. **Fukuda R, et al.** 2010. Vertebral osteomyelitis associated with Granulicatella adiacens. Tokai J. Exp. Clin. Med. **35**:126–129.
11. **Hepburn MJ, Fraser SL, Rennie TA, Singleton CM, Delgado B, Jr.** 2003. Septic arthritis caused by Granulicatella adiacens: diagnosis by inoculation of synovial fluid into blood culture bottles. Rheumatol. Int. **23**:255–257.
12. **Koligi K, et al.** 2011. Of bugs and joints. Oligoarthritis caused by Tropheryma whipplei. Internist (Berl.) **52**:884–888.
13. **Kommedal O, Karlsen B, Saebo O.** 2008. Analysis of mixed sequencing chromatograms and its application in direct 16S rRNA gene sequencing of polymicrobial samples. J. Clin. Microbiol. **46**:3766–3771.
14. **Kuhn C, et al.** 2011. Evaluation of commercial universal rRNA gene PCR plus sequencing tests for identification of bacteria and fungi associated with infectious endocarditis. J. Clin. Microbiol. **49**:2919–2923.
15. **Lange U, Teichmann J.** 2003. Whipple arthritis: diagnosis by molecular analysis of synovial fluid—current status of diagnosis and therapy. Rheumatology **42**:473–480.
16. **Mancini N, et al.** 2010. The era of molecular and other non-culture-based methods in diagnosis of sepsis. Clin. Microbiol. Rev. **23**:235–251.

17. **Nasim A, Baqi S, Akhtar SF.** 2011. Pseudomonas aeruginosa endocarditis in renal transplant recipients. Transpl. Infect. Dis. [Epub ahead of print.] doi:10.1111/j.1399-3062.2011.00667.x.
18. **Prassler R, Kempmann T, Vierling P.** 2008. Whipple's disease with segmental lesions in the proximal small intestine. Dtsch. Med. Wochenschr. **133**:460–463.
19. **Puechal X, Fenollar F, Raoult D.** 2007. Cultivation of Tropheryma whipplei from the synovial fluid in Whipple's arthritis. Arthritis Rheum. **56**:1713–1718.
20. **Riede U, Graber P, Ochsner PE.** 2004. Granulicatella (Abiotrophia) adiacens infection associated with a total knee arthroplasty. Scand. J. Infect. Dis. **36**:761–764.
21. **Sancho-Tello S, et al.** 2011. Performance of the LightCycler SeptiFast test Mgrade in detecting microbial pathogens in purulent fluids. J. Clin. Microbiol. **49**:2988–2991.
22. **Sontakke S, Cadenas MB, Maggi RG, Diniz PP, Breitschwerdt EB.** 2009. Use of broad range16S rDNA PCR in clinical microbiology. J. Microbiol. Methods **76**:217–225.
23. **Wellinghausen N, et al.** 2009. Diagnosis of bacteremia in whole-blood samples by use of a commercial universal 16S rRNA gene-based PCR and sequence analysis. J. Clin. Microbiol. **47**:2759–2765.
24. **Yager JE, et al.** 2010. Ureaplasma urealyticum continuous ambulatory peritoneal dialysis-associated peritonitis diagnosed by 16S rRNA gene PCR. J. Clin. Microbiol. **48**:4310–4312.
25. **Yanagihara K, et al.** 2010. Evaluation of pathogen detection from clinical samples by real-time polymerase chain reaction using a sepsis pathogen DNA detection kit. Crit. Care [Epub ahead of print.] doi: 10.1186/cc9234.
26. **Zimmerli W, Trampuz A, Ochsner PE.** 2004. Prosthetic-joint infections. N. Engl. J. Med. **351**:1645–1654.