Evaluation of the Alkaline Wash/Lysis Procedure for the Molecular Diagnosis of a Positive Bacterial Blood Culture in Clinical Routine Practice

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Blood culture is commonly used to detect microorganisms in patients with a suspected blood infection. This study evaluated the alkaline wash/lysis procedure to extract DNA of microorganisms in a clinical blood culture. A multiplex polymerase chain reaction (PCR) targeting the 16S rDNA (ribosomal DNA) gene and the fungal ITS (internal transcribed spacer) gene was used as a reliable indicator for the presence of microorganism DNA in the extracts. A total of 535

BacT/ALERT positive blood culture bottles were evaluated. Multiplex PCR showed positive results in 530 DNA extracts, but 5 DNA extracts gave negative results. We conclude that the alkaline wash/lysis procedure in combination with the multiplex PCR is a simple and sensitive method, which can be used in a standard diagnostic laboratory to detect microorganisms in blood culture material. *J. Clin. Lab. Anal.* 24:139–144, 2010. © 2010 Wiley-Liss, Inc.

Key words: blood culture; alkaline wash; lysis procedure

INTRODUCTION

Bacteremia is a serious condition with an overall in-hospital mortality above 20% (1–3). Rapid and reliable detection of microorganisms from blood is one of the most critical functions of a diagnostic microbiology laboratory (1,4). Automated blood culture systems with continuous monitoring not only reduce the time required to detect positive cultures, but also decrease specimen handling (2,3,5). BacT/Alert (bioMérieux, Lyon, France) is an automated system used for the incubation and detection of positive blood cultures (6,7).

Molecular-based diagnostic approaches are now more frequently used alongside conventional microbiological techniques in a diagnostic clinical microbiology laboratory because this technology has the potential to deliver rapid, specific, and sensitive detection of microbial pathogens in blood cultures within one working day (8–11). PCR amplification of regions of the 16S rRNA (12), 18S rRNA genes (13,14), and internal transcribed spacer (ITS) region (15) are such molecular-based approaches widely used in the identification of bacteria and fungi. It is essential, however, that the DNA extraction procedure produces DNA of sufficient

quality for PCR where the presence of PCR inhibitory substances, for example, sodium polyanethanesulfonate (SPS) found in blood culture media, can be successfully removed (16,17). To fit into a busy diagnostic laboratory, this extraction procedure must be relatively simple, fast, and sensitive, yet robust, reproducible, and cost-effective.

Previous studies indicated that the alkali wash/lysis procedure was a sensitive, reliable, and cost-effective method for extracting microbial DNA and removing PCR inhibitors from blood culture material, as well as an important fundamental prerequisite to later mole-

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cular analyses, including nucleic acid amplification techniques (18–20). But the alkali wash/lysis procedure has not been previously evaluated to prepare bacterial DNA from clinical blood culture specimens in clinical routine practice. The aim of this study was to validate the alkali wash/lysis procedure for clinical blood culture fluids. In addition, we developed a new and cost-effective multiplex PCR to detect bacteria or fungi from 535 extracted DNA by alkali wash/lysis procedure, to determine whether this technology holds promise for the direct examination of clinical specimens.

MATERIAL AND METHOD

Reference Organisms

The reference strains used in this study were *C. albicans* ATCC 76615, *C. parapsilosis* ATCC 10233, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 15545, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923. All strains were stored at -20°C in glycerol medium.

Clinical Samples and Controls

This study was conducted in the Clinical Microbiology Laboratory of the Tri-Service General Hospital from April to March 2007. A total of 5,491 blood culture sets were inspected during this period. Once the bottles had been received in the laboratory, they were placed into BacT/Alert blood culture system (bioMérieux, Marcy l'Etoile, France) and incubated until a positive signal was obtained from the instrument, or for a maximum of 7 days. Whenever there was a sign of microbial growth, an aliquot of blood–broth mixture was removed with a sterile needle and syringe. A portion was used for Gram staining and the remainder was subcultured onto a set of selective and nonselective media. Subsequent isolation, identification, and susceptibility testing of the organism were performed according to the standard operating procedures of the laboratory. In this study, the presence of microorganisms was observed in 535 cultures (BacT/Alert[®] SA [$n = 147$], BacT/Alert[®] SN [$n = 137$], BacT/Alert[®] FA [$n = 143$], and BacT/Alert[®] FN [$n = 108$]; Organon Teknina Corp., Durham, NC) taken from the 5,491 blood culture sets during this period (Table 1). After the presence of microorganisms was confirmed by Gram stain, the bottle was collected and nucleic acid extracted using the alkali wash/lysis procedure described below.

Alkali Wash/Lysis Procedure

Five hundred thirty-five bottles containing species of bacteria or fungi were extracted by the alkali wash/lysis procedure and analyzed by one-tube multiplex PCR for

the presence of bacterial 16S rDNA gene or fungal ITS gene. The method used in this investigation was from the study by Millar et al. (19). All DNA extracts were subsequently tested in duplicate by multiplex PCR using established in-house master mixture. Bacterial and fungal DNA was extracted from a 0.2 ml aliquot of blood culture fluid in 1 ml of alkali wash solution (0.5 M NaOH, 0.05 M sodium citrate; Sigma, St. Louis, MO). After gentle mixing for 10 min at room temperature on a rotary wheel, the mixture was centrifuged at $13,000 \times g$ for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml H_2O and centrifuged at $13,000 \times g$ for a further 5 min. The supernatant was discarded and the pellet was resuspended in 0.4 ml Tris–HCl–EDTA buffer (10 mmol/l Tris–HCl, 1 mmol/l EDTA, pH 8.0) containing 10 U of lyticase (Sigma-Aldrich, Steinheim, Germany). Tubes were incubated at 30°C for 30 min, and then added 0.1 ml of 10% SDS incubated at 60°C for 60 min.

Specificity and Sensitivity of Multiplex PCR

Here, the specificity and sensitivity of the multiplex PCR were estimated with 10-fold dilutions of standard amounts of DNA of *C. albicans* ATCC 76615, *C. parapsilosis* ATCC 10233, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 15545, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923. The lowest limit of detection of bacterial cells or purified DNA by multiplex PCR was determined using ATCC strains. Hundred nanogram of DNA was 10-fold serially diluted in sterile distilled water. Ten fold serially diluted DNA was added directly to the multiplex PCR mixture. All PCR reactions were performed in triplicate.

PCR Amplification and Detection

The broad-range bacterial specific primer pair Bact 5F and Bact 809R (21) (Table 2) were used to amplify a portion of the 16S rRNA gene (corresponding to the gene positions 5 to 809 of the *Escherichia coli* 16S rRNA gene), and generate 804 bp PCR product. The broad-range fungal-specific primer pair: Fungi ITS1 and Fungi ITS4 (12,22), which bind to conserved regions, with corresponding positions to *Saccharomyces cerevisiae* 18S (1,769–1,787) and 28S (41–60), amplify an approximately 500 bp product that encompasses the entire ITS1, 5.8S, and ITS2 rRNA gene regions. These two sets of primers were used in the one-tube multiple PCR mixture. A bacterial strain was expected to produce an 804 bp product. A fungal strain was expected to produce a 400–900 bp product. PCR was performed in a total volume of 50 μl , containing 250 μM of each dNTP, 2.0 mM MgCl_2 , 0.5 U of DyNAzyme EXT (Finnzymes Oy, Espoo, Finland), 5 μM of each primer,

TABLE 1. Species Isolated from Blood Cultures in this Study

Species (Gram-smear categories ^a)	Positive bottle types ^b				Total
	SA	SN	SF	SG	
<i>Abiotrophia defectiva</i> (GPC)			1	1	2
Coagulase Negative Staphylococcus (GPC)	20	19	35	31	105
<i>Enterococcus</i> spp.			1	1	2
<i>Enterococcus faecalis</i> (GPC)			3	4	7
<i>Enterococcus faecium</i> (GPC)			2	3	5
<i>Enterococcus gallinarum</i> (GPC)			1	1	2
<i>Micrococcus</i> spp. (GPC)	1	1	1	1	4
<i>Staphylococcus aureus</i> (GPC)	13	15	24	25	77
<i>Staphylococcus saprophyticus</i> (GPC)			1		1
<i>Streptococcus pneumoniae</i> (GPC)	4	3			7
<i>Viridans streptococcus</i> (GPC)		2	1	2	5
<i>Bacillus</i> spp (GPB)		1			1
<i>Corynebacterium diphtheriae</i> (GPB)	2	1	1		4
<i>Exiguobacterium</i> spp. (GPB)	1				1
<i>Lactobacillus</i> spp. (GPB)	1	1			2
<i>Acinetobacter baumannii</i> (GNB)			3		3
<i>Acinetobacter</i> spp. (GNB)	1		8	1	10
<i>Aeromonas hydrophila</i> (GNB)	1	1	1	1	4
<i>Enterobacter cloacae</i> (GNB)	4	4	6	7	21
<i>Enterobacter aerogenes</i> (GNB)			1		1
<i>Enterobacter agglomerans</i> (GNB)		1		1	2
<i>Escherichia coli</i> (GNB)	50	51	7	6	114
<i>Flavobacterium indologenes</i> (GNB)	1				1
<i>Haemophilus influenza</i> (GNB)	1				1
<i>Klebsiella pneumoniae</i> (GNB)	24	24	11	10	69
<i>Morganella morganii</i> (GNB)				1	1
<i>Proteus mirabilis</i> (GNB)	3	2			5
<i>Pseudomonas aeruginosa</i> (GNB)	5	1			6
<i>Pseudomonas fluorescens</i> (GNB)			1		1
<i>Pseudomonas oryzae</i> (GNB)	2				2
<i>Salmonella</i> group B (GNB)		1			1
<i>Salmonella</i> group D (GNB)			1		1
<i>Serratia liquefaciens</i> (GNB)			1	1	2
<i>Sphingomonas paucimobilis</i> (GNB)	2				2
<i>Stenotrophomonas maltophilia</i> (GNB)	2		1		3
<i>Mycobacterium abscessus</i>	2				2
<i>Clostridium perfringens</i> (GPB)		1			1
<i>Propionibacterium acnes</i> (GPB)		1			1
<i>Bacteroides fragilis</i> (GNB)		1			1
<i>Bacteroides vulgatus</i> (An GNB)		2			2
<i>Veillonella alcalescens</i> (GNC)				2	2
<i>Enterococcus faecalis</i> (GPC)+ <i>Citrobacter freundii</i> (GNB)			1	1	2
<i>Enterococcus faecalis</i> (GPC)+ <i>Pseudomonas aeruginosa</i> (GNB)			4	4	8
<i>Enterococcus faecium</i> (GPC)+ <i>Acinetobacter baumannii</i> (GNB)			1	1	2
<i>Escherichia coli</i> (GNB)+ <i>Serratia marcescens</i> (GNB)			2	2	4
<i>Klebsiella pneumoniae</i> (GNB)+ <i>Clostridium perfringens</i> (GPB)	1	1			2
<i>Klebsiella pneumoniae</i> (GNB)+ <i>Serratia marcescens</i> (GNB)			1	1	2
<i>Pseudomonas aeruginosa</i> (GNB)+B- <i>Streptococcus</i> non ABD (GPC)	2	2			4
<i>Staphylococcus aureus</i> (GPC)+B- <i>Streptococcus</i> group B (GPC)	1	1			2
<i>Candida albicans</i> (Yeast)	2		11		13
<i>Candida glabrata</i> (Yeast)			1		1
<i>Candida tropicalis</i> (Yeast)			10		10
Total microorganisms	147	137	143	108	535

^aGram-smear categories: GPC, Gram-positive cocci; GPB, Gram-positive bacilli; GNB, Gram-negative bacilli; GNC, Gram-negative cocci.^bPositive bottle types: SA, standard aerobic bottle; SN, standard anaerobic bottle; SF, aerobic FAN bottle; SG, anaerobic bottle.

TABLE 2. Broad-Range Primers Used for PCR Amplification of Regions of the Bacterial 16S rRNA Gene and the Fungal Internally Transcribed Spacer Gene (ITS)

Target	Primer name	Sequence 5'–3'
16S rRNA	Bact 5F	GAA GAG TTT GAT CMT GGC TC
	Bact 809R	GCG TGG ACT ACC AGG GTA TC
ITS	Fungi ITS1	TCC GTA GGT GAA CCT GCG G
	Fungi ITS4	TCC TCC GCT TAT TGA TAT GC

and 5 µl (50 ng) of template DNA in 1 × EXT buffer. Temperature cycling conditions were as follows: 96°C for 5 min, followed by 35 cycles of 96°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis on 1.5% agarose or 6% polyacrylamide gels. Molecular sizes of the products were estimated by comparing the migration distances of DNA bands of a 100-bp DNA ladder.

RESULTS

Specificity and Sensitivity of Multiplex PCR

The specificity and sensitivity of multiplex PCR were estimated with 10-fold dilutions of standard amounts of DNA of *C. albicans* ATCC 76615, *C. parapsilosis* ATCC 10233, *C. tropicalis* ATCC 750, *C. glabrata* ATCC 15545, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923. Final products of the multiplex PCR were analyzed by 1.5% agarose gel electrophoresis. The multiplex PCR assay had detection limits of 0.1 pg bacterial DNA and 0.5 pg fungal DNA analyzed by 10-fold serial dilution test. No cross-reactivity between the bacterial broad-range primers and the fungal broad-range primers was observed. The identity of each multiplex PCR product was further confirmed by DNA sequencing. No false-positive multiplex-PCR signals were observed for the 10 culture negative specimens applied with the alkali wash/lysis procedure (data not shown).

Assessment of the Specificity of the Multiplex PCR Method and the Performance of the DNA Extraction Methods Used

The presence of pathogenic microorganisms was observed in 535 cultures taken from the 5,491 blood culture sets during the period. Two hundred nineteen contained only a Gram-positive cocci, 260 contained only a Gram-negative bacilli, 10 contained only a Gram-positive bacilli, 2 contained only a Gram-negative cocci, 24 contained only yeast, 16 contained both a Gram-positive cocci and a Gram-negative bacillus, 2 contained both a Gram-positive bacillus and a Gram-negative bacillus, and 2 contained only AFB positive bacillus. All

TABLE 3. The Result of Multiplex PCR for DNA Extracts from the Alkaline Wash/Lysis Procedure

Bottle type	Culture result vs. multiplex PCR result			
	Bacteria		Fungi	
	Match	No PCR product	Match	No PCR product
FA (143)	121		19	3 ^c
FN (108)	107	1 ^a	0	0
SA (147)	144	1 ^b	2	
SN (137)	137		0	0
All four bottles (535)	509	2	21	3

^aCoagulase Negative *Staphylococcus*.

^b*Corynebacterium diphtheriae*.

^c*Candida albicans* (1), *Candida glabrata* (1), and *Candida tropicalis* (1).

535 bottles were evaluated by the alkali wash/lysis procedure, tested, and categorized as having originated from bacteria or fungi on the basis of this multiplex PCR assay (Table 3). Specific PCR products were detected in 530 of 535 positive cultures (99.07%) by using DNA prepared by the alkali wash/lysis procedure. Based on the multiplex PCR results, 509 of the isolates belonged to the bacteria and the other 21 isolates were fungus isolates. The results of multiplex PCR method were 100% concordant with conventional identification of the isolates grown from subculture. Five of the 535 DNA extracts by the alkali wash/lysis procedure failed to generate a PCR product in this study. There were three extracts of fungi (*Candida albicans*, *C. glabrata*, and *C. tropicalis*) and two extracts of bacteria (Coagulase Negative *Staphylococcus* and *Corynebacterium diphtheriae*).

DISCUSSION

Blood culture is one of the most important laboratory tests used for the prompt diagnosis and effective management of septicemia (2,3). Early administration of appropriate empirical antibiotic treatment has repeatedly been associated with improved survival in patients with bacteremia; yet up to 40% of all patients with bacteremia receive inadequate antibiotic treatment until the first notification of a positive blood culture (1–3). Because molecular-based diagnostic methods are now becoming established in clinical laboratories, coupling this technology has provided a rapid, specific, and sensitive detection of microbial pathogens from blood cultures within one working day (11,20).

Previous studies have shown that the broad-range bacterial primers and broad-range fungal primers used in their study were specific and sensitive for the detection

of bacteria and fungi (12,21,22). The major problem that hinders the molecular investigation of blood culture material involves the inhibiting agents of PCR amplification (20,22). All blood culture materials contain anticoagulant, anti-complementary agents, and sodium polyanetholesulfonate (SPS). Previous studies (17,19) suggest that SPS may be the main inhibitory agent in blood culture material. Several factors in blood from patient also reduce the PCR amplification efficiency, such as heme (23), IgG (24), high concentrations of leukocyte DNA, and other inhibitors and other unknown factors (16,25).

Many DNA extraction methods have been evaluated in previous studies to ascertain the most suitable method to isolate the high-quality DNA, which could be used further in the molecular analysis (12,18–20,22). Some of the commercial kits, such as the QIAmp blood kit (Qiagen Corporation, Valencia, CA) and the Roche High Pure PCR Template Preparation Kit (Roche, Rotkreuz, Switzerland), with procedures to remove PCR inhibitors, are reported to be ineffective and labor-intensive with regard to DNA extraction from blood cultures (17,19,22). In this study, we applied the alkaline wash/lysis procedure to prepare the samples that are used later in a multiplex PCR, targeting the broad-range bacteria 16S rDNA gene and the fungal ITS gene. Using the alkaline wash/lysis procedure with the multiplex PCR assay in this study, the sensitivities for total samples, bacteria and fungi are 99.06% (530/535), 99.6% (509/511), and 87.5% (21/24), respectively. Five positive blood cultures were false-negative with PCR. These negativities had been verified by applying the other broad-range 16S rDNA and 18S rDNA primers in separate PCR. The effects of the PCR inhibitors could not be ruled out in the five cases. The efficiency limitation of our digestion procedure was therefore presumed. However, its efficiency may be improved by adding more enzyme or extending the digestion time. Three *Candida*-positive blood cultures were false-negative with PCR. This suggests that the fact that extracting DNA from certain fungi by this alkaline wash/lysis procedure yielded less DNA may be due to insufficient cell lysis, as shown in a previous study (26).

This alkaline wash/lysis procedure combined with molecular-based diagnosis may be used for two additional applications: false culture positive bottles and platelet concentrates contaminated with bacteria. Various studies have shown that about 1–10% of the samples processed by these automated systems give a positive result and subsequent Gram stain and subculture on standard media yield no bacteria (27). Those uncertainties that cause false-positive culture bottles include: elevated leukocyte count of the patient, fastidious organisms that are not cultivable on standard

media, and autolysis of particular organisms (ex.: *Strep. Pneumoniae*) (12,28). The bacterial contamination of platelet concentrates remains a persistent but often ignored problem in transfusion medicine (29,30). Automated bacterial blood culture systems meet many of the requirements of an ideal test, but have also been proven to be inconvenient because of the time required to indicate the presence of bacteria, their low specificity, and their lack of sensitivity when the initial levels of contaminating bacteria are low (29,30). In this study, the protocol of the alkaline wash/lysis procedure combined with multiplex PCR assay allowed for the detection of false-positive blood culture and low amounts of contaminated bacteria in platelet concentrates.

To extract DNA from the clinical blood culture is a critical process in detecting and identifying pathogens by PCR. Our results demonstrate that the alkaline wash/lysis procedure is a useful and optimal tool for routine DNA extraction from positive blood cultures.

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