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TARGET ENRICHED MULTIPLEX PCR (TEM-PCR™) FOR RAPID DETECTION OF BLOODSTREAM INFECTIONS

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OVERVIEW

Bloodstream infection is a leading cause of morbidity and mortality in intensive care units (ICUs). The standard detection method for sepsis is phenotypic identification of blood infection using blood culture and Gram staining, but the practical value is impaired by the delayed turnaround time and low sensitivity of slow-growing, fastidious organisms. Molecular diagnostics for bacterial identification can provide increased sensitivity and rapid detection. In this chapter, we describe how Target Enriched Multiplex PCR (TEM-PCR™) can be applied for the detection of bloodstream pathogens. This technology provides rapid results with high sensitivity and specificity in blood samples, capable of detecting up to two dozen organisms in a single reaction. Studies have shown that implementation of molecular diagnostics, such as TEM-PCR, for blood infections significantly reduced the average turnaround time for clinical intervention, decreased length of stay in the ICU, and lowered the cost of antimicrobial treatment. We examined 464 positive blood culture samples using the Diatherix Infectious Disease Panel (IDP). Data suggested the most prevalent organism is *Enterococcus faecalis*, which was contrary to reports that the most commonly detected organism in blood culture was *Staphylococcus aureus*. This result led us to hypothesize that blood culture media may contain residual DNA and can produce false-positive results when using molecular diagnostics. We confirmed this hypothesis after performing microbial testing and TEM-PCR analysis on sterile, un-inoculated media from multiple lots of blood culture bottles. No growth was observed in media, but bacterial DNA was detected in each sample. These data demonstrate that blood culture bottles are unsuitable for molecular detection of bloodstream pathogens. Alternative devices are in production for bacterial identification directly from blood samples, and the combination of TEM-PCR technology with these methods would provide increased sensitivity and rapid results for faster and more accurate treatment of septic patients.

Bloodstream infection is a leading cause of morbidity and mortality in intensive care units, affecting more than one million Americans annually. The mortality rate associated with bloodstream infection ranges from 10 to 40% and has aggregate healthcare costs of approximately $16 billion a year *(1, 2)*. A rapid (within 24 h) and accurate identification of a broad range of microbial or fungal pathogens is the key for successful management of patients with bloodstream infection. Blood culture is still considered the gold standard in the detection of bloodstream infection, requiring the culture of patient blood in an automated, continuously monitored system followed by Gram staining and subculturing. The practical value of blood culture in the diagnosis of sepsis is impaired by the delay of time-to-results and low sensitivity of slow-growing and fastidious organisms. Every hour of delay for patient treatment of suspected septic conditions results in an increase in patient mortality of 7% *(3)*. Thus, early and accurate detection of blood pathogens will greatly benefit patient care.

Molecular diagnostic methods using real-time polymerase chain reaction (PCR) are used routinely for pathogen detection in clinical settings *(4–6)*. Recently, several molecular approaches have been developed to identify a large spectrum of pathogens and antibiotic resistances in a single sample. In this chapter, we describe how target enriched multiplex PCR (TEM-PCR) can be applied for the detection of bloodstream pathogens and the challenges associated with application of multiplex PCR-based tests for testing positive blood culture bottles.

TEM-PCR

The TEM-PCR methodology was first reported in 2006 for simultaneous amplification and identification of 25 human papillomavirus types *(7)*. This technology was commercialized by Qiagen *(8, 9)* and now is used by Diatherix Laboratories (Huntsville, AL, US) as laboratory-developed tests in a high-complexity CLIA (Clinical Laboratory Improvement Amendments)-certified laboratory *(10)*. TEM-PCR provides sensitive and specific identification of pathogenic organisms with short time-to-answer. This technology platform has proven to be flexible for inclusion of novel targets (in case of emerging

epidemics) and can be adapted to different detection platforms, such as bead- or microarray-based systems.

A single TEM-PCR reaction can contain up to 100 individual primers, with up to five primers being used for each target. The cycling conditions are optimized to achieve high specificity for target amplification with minimal interactions between primers during the two main steps of the process: target enrichment and exponential amplification of amplicons generated during target enrichment. Two pairs of nested gene-specific primers (*Fo,* forward out; *Fi,* forward in; *Ri,* reverse in; *Ro,* reverse out) are designed for each target and are included in the reaction (Figure 22-1A). These primers are present at extremely low concentrations and are primarily used during the target enrichment step, consisting of few PCR cycles. The purpose of using two pairs of target-specific primers, instead of the standard single pair of forward and reverse primers, is to increase target-specific DNA yield that will be used for downstream exponential amplification. *Fi* and *Ri* primers are designed with a unique tag sequence that can be recognized by a universal set of primers, called SuperPrimers, which are also included in the same PCR reaction. SuperPrimers are present at a concentration necessary for asymmetric PCR amplification, where the final products of amplification are represented by biotin-labeled, single-stranded DNA derived from the biotin-labeled reverse SuperPrimer. The flexibility of TEM-PCR comes from coupling target-specific complementary detection probes to carboxyl groups on any bead- or glass-based substrate (Figure 22-1B), thus making this technology amendable to perform high-throughput testing of clinical samples as an automatable laboratory procedure.

LIMITATIONS OF BLOOD CULTURE-BASED DETECTION OF BLOODSTREAM INFECTIONS

The current standard for identification of bloodstream infection in the hospital setting involves phenotypic identification of microorganisms from positive blood culture bottles. Although the limitations of this approach are widely discussed *(11)* and understood by the clinical community, there is a lack of clinically accepted alternative diagnostic methods that would replace blood culture-based detection of infections. Fully automated blood culture systems, such as BACTEC (Becton Dickinson, Sparks, MD, US) and BacTAlert (Biomerieux, Durham, NC, US), are routinely used in clinical laboratories. The growth of organisms is monitored by measuring the production of CO_2 with colorimetric (BacTAlert) or fluorescent sensors (BACTEC). The main inherent limitation is the long time-to-answer, where timing is critical for patient treatment and outcome. Under normal circumstances, a positive blood culture is identified by an automated microbial detection system within 8 to 36 h from the time of blood collection. From that point, it can take 12 to 48 h to initially classify the pathogen species and another 24 to 48 h to report the final pathogen identification and susceptibility profile. Recent technological developments in this area introduced new liquid growth media, resins that absorb antibiotics, and new fluorescent or colorimetric sensors to shorten detection time. Nevertheless, the median time to determine positivity of blood cultures is still 15 h, and the total time until full identification (including antibiotic susceptibility) is more than 72 h for bacteria and more than 60 h for fungi *(12, 13)*. According to guidelines from the Infectious Diseases Society of America, the

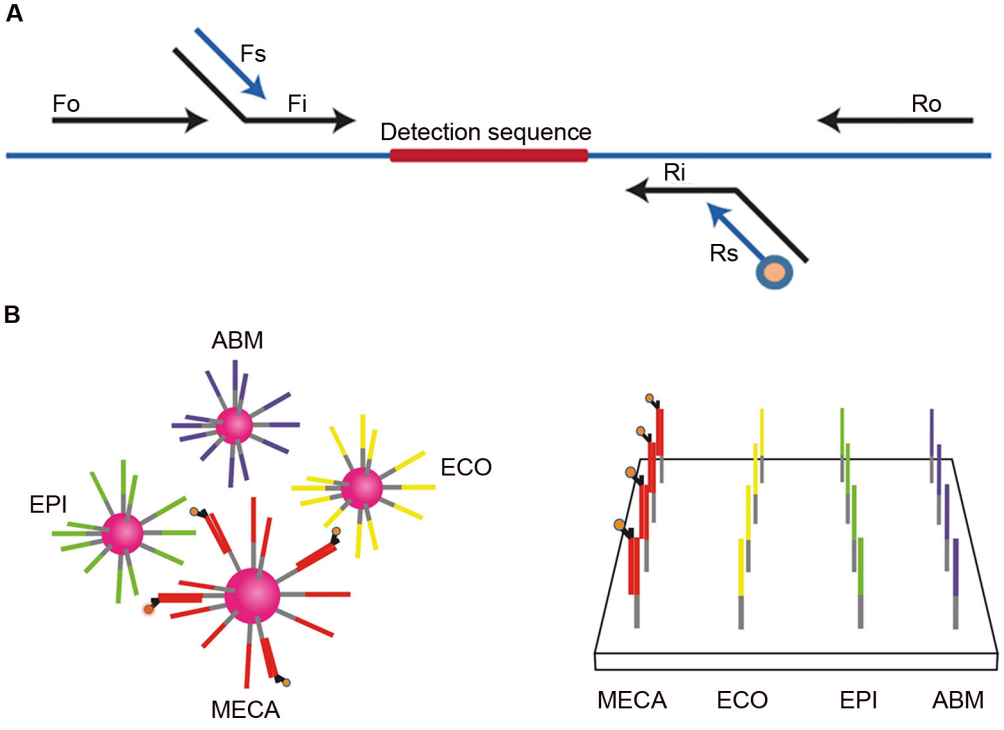

FIGURE 22-1 TEM-PCR amplification (A) and detection of target-specific amplicons (B).

lack of rapid technology for identification of pathogens during the first 24 h of hospital admission of a septic patient requires physicians to administer broad-spectrum antimicrobial therapy before blood samples are collected. Prescribing broad-spectrum antimicrobials before reports of final culture and susceptibility testing has proven to reduce mortality and healthcare costs, but this approach is contributing to an increase of antibiotic resistance in hospitals.

Blood cultures are capable of detecting as low as one colony-forming unit (CFU) of bacteria or fungi/yeast per 10 mL of blood. Typically, two to three blood culture sets are collected per patient with 20 to 30 mL of blood per set. It has been shown that increased blood volume and number of culture sets correspond to higher pathogen detection rates *(14)*; however, extracting such volume of blood is not feasible in children and neonates and can result in false-negative results for pediatric septic patients *(15)*. Many variables play a role in the final identification of a bloodstream infection, including the time from sample collection and incubation, the system used for culturing, the blood volume, the type of pathogen present, its optimal growth conditions, and the presence of antibiotics in the blood. In addition, our study has shown that media in blood culture bottles contain residual DNA that could produce false-positive results when molecular tests are being used for pathogen identification.

TABLE 22-1 LIST OF ORGANISMS IN INFECTIOUS DISEASE PANELS AND *STAPHYLOCOCCUS* DIFFERENTIATION PANEL

Organism/antibiotic resistance	TEM-PCR panel	Target name
Acinetobacter baumannii	IDP	ABM
Enterobacter aerogenes	IDP	EAER
E. cloacae	IDP	ENCL
Enterococcus faecalis	IDP	EFLS
E. faecium	IDP	EFCM
Escherichia coli	IDP	ECO
Klebsiella pneumoniae	IDP	KPN
Proteus mirabilis	IDP	PROM
Pseudomonas aeruginosa	IDP	PMA
Serratia marcescens	IDP	SMA
Staphylococcus aureus	SDP, IDP	NUC
S. epidermidis	SDP, IDP	EPI
Staphylococcus, coagulase-negative	SDP, IDP	TUF
S. aureus, methicillin resistant	SDP, IDP	MECA
Streptococcus pyogenes	SDP, IDP	SPY
Stenotrophomonas maltophila	IDP	XMA
Aminoglycoside-resistance	SDP, IDP	AACA
Erythromycin/clindamycin resistance	SDP, IDP	ERMA/ERMC
Panton-Valentine leukocidin toxin	SDP, IDP	PVL
Tetracycline resistance	SDP, IDP	TETK/TETM
Vancomycin resistance	SDP, IDP	VANA/VANB

IDP = Infectious Disease Panel; SDP = *Staphylococcus* Differentiation Panel; TEM-PCR = Target Enriched Multiplex PCR.

TESTING POSITIVE BLOOD CULTURES ON TEM-PCR PANELS

Diatherix has developed several TEM-PCR-based panels for the detection of gram-positive and gram-negative bacteria in clinical samples (Table 22-1). Tang and colleagues *(16)* described how an earlier version of a TEM-PCR-based *Staphylococcus* Differentiation Panel (SDP) can be applied for staphylococcal identification, detection of Panton-Valentine leukocidin (PVL), and antibiotic resistance determinants in positive blood culture bottles. Phenotypic identification of organisms was compared with the results obtained using molecular methods. Authors reported that the overall accuracy for molecular detection of culture-negative staphylococci, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *S. aureus*, and non-staphylococci was 96.7, 92.1, 72.5, and 66.7%, respectively, in comparison with phenotypic-based microbial identification. Although concordance between detection of *aacA, ermA,* and *ermC* with antimicrobial susceptibility profiles for gentamicin, erythromycin and clindamycin was relatively poor, all specimens with detected MRSA (n = 39) did not contain any *ermA* or *ermC* genes, resulting in zero major errors and a positive predictive value of 100%.

Eiland et al. *(17)* conducted a randomized study at a large 800+ bed hospital to evaluate if the implementation of molecular tests for pathogen detection in positive blood culture bottles can affect earlier intervention for antibiotic coverage and improve clinical outcome of patients with staphylococcal bloodstream infections. Both culture-based and TEM-PCR results were compared. Authors reported that the utilization of TEM-PCR *Staphylococcus* Differentiation Panel for positive blood culture bottles significantly reduced the average turnaround time for clinical intervention (50.65 h for culture-based testing and 11.78 h using TEM-PCR). The concordance between the two methods on bacterial identification specificity and sensitivity was 93 and 80%, respectively. In addition, TEM-PCR-based data produced optimized and deescalated therapy in 27 and 23% cases, respectively, which resulted in decreased length of stay in the ICU by an average of 3 days and lowered cost of antimicrobial treatment. Although this study was completed using a small sample size, limiting the opportunity for extensive data mining, data indicate a statistically significant decrease in the turnaround time and length of stay for patients using molecular diagnostics vs culture-based testing. Results suggested that the implementation of TEM-PCR-based panels for pathogen identification in positive blood culture bottles was very promising and warranted future investigations with a larger sample size.

SURPRISE FINDING: POWER OF MULTIPLEX PCR APPROACH

A collaborative study with three large hospitals in Oklahoma, Ohio, and Alabama was initiated to explore the potential impact of implementation of the TEM-PCR IDP for rapid bacterial identification in positive blood culture samples. The goal of this study was to test a minimum of 50 positive blood culture bottles collected from septic patients for the presence of bacterial organisms that can be detected in an IDP (see Table 22-1). It was previously reported that a pathogen was not identified in at least one third of septic cases because of problems associated with blood cultures and empiric antibiotic treatment *(18)*, and

the identification of the type of organism can be an important determinant of morbidity and mortality *(19, 20).* Bloodstream infections caused by gram-positive organisms have increased in frequency over the years, likely because of a greater use of invasive procedures and an alarming rise of hospital-acquired infections *(18, 21).* The percentage of polymicrobial infection as well as the proportion of multidrug-resistant bacteria such as *Pseudomonas* and methicillin-resistant Staphylococci has also significantly increased over time *(22).* Thus, it is important to employ a comprehensive molecular panel for the detection of gram-positive and gram-negative organisms, such as IDPs, for testing of positive blood culture bottles.

Positive blood culture bottles (n = 464) were collected over a 5-month period. Blood from septic patients was collected in BacTAlert blood culture bottles (Biomerieux). Bottles were incubated on-site at hospitals between 1 and 72 h until shipment to Diatherix Laboratories (Huntsville, AL, US) and were tested with TEM-PCR IDP (Table 22-2). In our study, the frequency of detection of gram-positive vs gram-negative organisms was disproportionally higher (28 vs 2.8%, respectively; Table 22-2). The predominant gram-positive bacteria detected in positive blood culture bottles were *Enterococcus faecalis* (13.4%), *Staphylococcus aureus* (11.6%), *S. epidermidis* (1.3%), and others (1.7%). Among gram-negative organisms, *Escherichia coli* had the highest detection rate at 1.7%, followed by *Proteus mirabilis* (1.1%) and *Klebsiella pneumoniae* (0.4%). The finding that *E. faecalis* was the most prevalent organism detected in positive blood culture bottles was surprising. It was also contradictory to numerous reports in which the most frequently detected organism in positive blood culture bottles is *S. aureus (19).* Because none of the vendors (Becton Dickinson or Biomerieux) supplied blood culture bottles free of residual nucleic acid, we suspected that some lots of blood cultures bottles used at different sites may contain residual DNA introduced during the manufacturing processes. In order to test this hypothesis, we collected nine different lots of blood culture bottles (BacTAlert, Biomerieux), four of which were used in our clinical study. Two aliquots of media were taken from sterile, un-inoculated blood culture bottles from each lot: one was for DNA extraction and follow-up analysis on the TEM-PCR IDP, and the other was for microbiological analysis of pathogen identity. The microbial culture results were negative for all 14 samples, confirming that all nine lots of blood culture bottles were sterile at the time of blood collection; however, culture media collected from six of 14 tested bottles (42.9%) tested positive for *E. faecalis* and *Enterobacter cloacae* with TEM-PCR. Other organisms from IDP were not detected. Thus, we cannot exclude the fact that predominant detection of *E. faecalis* in samples from this clinical study could be a result of residual contaminant DNA present in sterile blood culture media. The contamination can occur at the manufacturer site as bottles are filled with unsterilized media for later sterilization. This process has no effect on the intended use of blood culture bottles because sterility of the bottles is not compromised; however, it makes blood culture bottles unsuitable for multiplex detection of bloodstream pathogens. With increased development and application of PCR approaches for identification of bloodstream pathogens, the presence of contaminant DNA in sterile products may hamper diagnostic accuracy of molecular methods *(23, 24).*

In order to identify a lot of blood culture bottles with minimal residual DNA from both manufacturers (Becton Dickinson and Biomerieux), we used real-time PCR with primers specific for 16S rDNA. This approach is widely used for detection of any bacterial species because some regions of 16S rDNA are highly conserved across the entire microbiome *(25).* Four pairs of primers targeting a conserved region of 16S rDNA were selected *(26, 27)* and tested for PCR efficiency (data not shown). One pair of primers with the lowest Ct (threshold cycle) values and a single melt peak using SYBR chemistry was chosen for the study. Sterile media (500 µL) from un-inoculated blood BACTEC and BacTAlert culture bottles was used in downstream processes. PCR inhibitors were removed, and DNA extraction with Omega Biotek DNA extraction kits (Marietta, GA, US) adapted on the KingFisher system (ThermoFisher, Waltham, MA, US) was performed. Positive (DNA extracted from *S. aureus*) and negative (water) controls were included through the DNA extraction process to confirm the process efficiency and absence of bacterial DNA contamination in extraction reagents. Extracted DNA samples were tested in duplicates by real-time PCR on a CFX96 real-time PCR cycler (BioRad, Pleasanton, CA, US) with SYBR chemistry. Representative examples of amplification curves are shown in Figure 22-2, and results of the testing are summarized in Table 22-3. Using real-time PCR with 16S primers, we detected the presence of bacterial DNA in all tested media from sterile, un-inoculated blood culture bottles from both vendors, with Ct values ranging from 21 to 26 (Figure 22-2). Next, we aimed to identify the bacterial DNA using two molecular approaches: real-time PCR with target-specific primers and TEM-PCR using an

TABLE 22-2 BACTERIAL TARGET IDENTIFICATION IN POSITIVE BLOOD CULTURE BOTTLES (n = 464) USING TEM-PCR INFECTIOUS DISEASE PANELS

Target	No. of positives	Target	No. of positives	Target	No. of positives	Target	No. of positives
TUF	2 (0.4%)	ERMA	6 (1.3%)	ABM	1 (0.2%)	KPN	2 (0.4%)
EPI	4 (0.9%)	ERMC	1 (0.2%)	EAER	0 (0%)	PMA	1 (0.2%)
NUC	15 (3.2%)	TETK	0 (0%)	ECO	8 (1.7%)	PROM	5 (1.1%)
MECA	23 (5%)	TETM	9 (1.9%)	EFCM	1 (0.2%)	SMA	0 (0%)
PVL	7 (1.5%)	VANA	0 (0%)	EFLS	62 (13.4%)	XMA	0 (0%)
AACA	1 (0.2%)	VANB	0 (0%)	ENCL	8 (1.7%)	SPY	0 (0%)

For abbreviations, see Table 22-1.

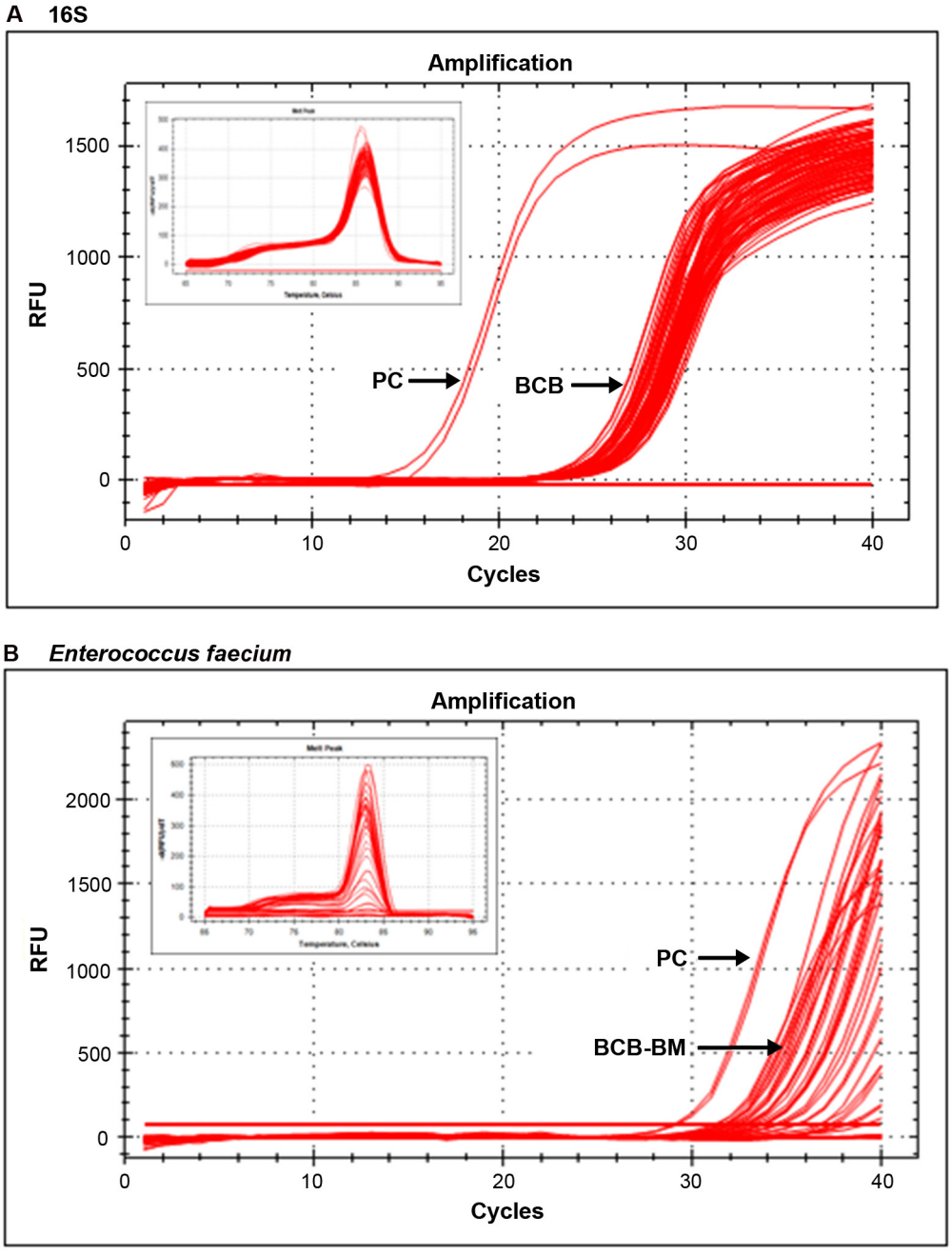

FIGURE 22-2 Real-time PCR amplification with SYBR chemistry and melt curve analysis for screening of blood culture media for a presence of residual bacterial DNA. Detection of 16S microbial rDNA in blood culture bottles (BCB, both vendors) and in positive control (PC) *Staphylococcus aureus* (A). The contaminant DNA from *Enterococcus faecalis* was detected in blood culture bottles from Biomerieux (BCB-BM). Positive control (PC) is DNA isolated from *E. faecalis* (B). Insets: melt curves for assays specific for 16S and *E. faecium*.

IDP and a staphylococcus Differentiation Panel (SDP). Using real-time PCR, we detected the presence of residual DNA from *Enterococcus faecium* (63.6%) and *E. faecalis* (4.5%) in BacTAlert blood culture media samples (Figure 22-2B and Table 22-3); these organisms were not detected in BACTEC bottles with real-time PCR. We expected that increased sensitivity with TEM-PCR would reveal more bacterial species because of the target enrichment step included prior to PCR amplification. In fact, with TEM-PCR, increased detection of *E. faecium* (100%; 22/22 samples) and *E. faecalis* (40.9%; 9/22) was identified in BacTAlert blood culture bottles. *E. faecium* was not detected in BACTEC bottles. *E. faecalis*, however, was detected in 9.1% of BACTEC bottles (2/22 samples). Using TEM-PCR, we also detected contaminant DNA from

TABLE 22-3 BACTERIAL TARGET IDENTIFICATION USING REAL-TIME PCR AND TEM-PCR IN STERILE UN-INOCULATED MEDIA COLLECTED FROM BACTEC (N = 22) AND BACTALERT BOTTLES (n = 22)

	TEM-PCR		qPCR	
Target	BACTEC	BacTAlert	BACTEC	BacTAlert
16S	ND	ND	22 (100%)	22 (100%)
EFCM	0 (0%)	22 (100%)	ND	14 (63.6%)
EFLS	2 (9.1%)	9 (40.9%)	0 (0%)	1 (4.5%)
PROM	3 (13.6%)	0 (0%)	0 (0%)	ND
XMA	1 (4.5%)	0 (0%)	0 (0%)	ND
EPI	0 (0%)	3 (13.6%)	ND	0 (0%)
NUC	0 (0%)	1 (4.5%)	ND	0 (0%)
TUF	0 (0%)	2 (9.1%)	ND	0 (0%)

ND = not determined; qPCR = quantitative polymerase chain reaction; TEM-PCR = Target Enriched Multiplex PCR. For other abbreviations, see Table 22-1. BACTEC (Becton Dickinson) and BacTAlert (Biomerieux). Targets were not examined with qPCR due to 0% detection with TEM-PCR.

other bacterial species. BacTAlert media contained DNA from *S. epidermidis* (13.6%) and *S. aureus* (4.5%), whereas DNA from *P. mirabilis* and *Stenotrophomonas maltophilia* was detected in BACTEC blood culture media (13.6 and 4.5%, respectively; Table 22-3). These targets were not detected by real-time PCR.

Various studies have shown that false positives were found in up to 10% of samples processed in automated blood culture systems and could not be confirmed by standard microbiological cell cultures *(23, 28, 29)*. There is an increased number of multiplex molecular diagnostic methods for testing positive blood culture bottles that are using PCR-based target enrichment prior to amplification *(30, 31)*. Although the concordance of molecular tests with conventional cell culture approaches are relatively good, when these tests are being used in a clinical setting, one should take into account the presence of residual contaminant DNA in sterile, un-inoculated blood culture media that can produce false-positive results and affect patient treatment. These problems could be avoided if molecular tests were performed directly on blood collected from septic patients. Novel molecular diagnostic devices are at various stages of commercialization and allow for pathogen enrichment directly from blood via multiple capture methodologies *(24, 32, 33)*. The combination of TEM-PCR with any of the pathogen capture methods has a significant potential for streamlining clinical tests and assisting physicians in making rapid and better informed therapeutic decisions for septic patients.

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