Rapid Identification of Sepsis Pathogens Using Real-Time Nested Multiplex PCR

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UPDATED ABSTRACT

Bacterial sepsis is a leading cause of death in the U.S. Delay in identification of pathogens, even after positive blood culture, significantly contributes to morbidity and mortality. A rapid multiplex diagnostic test could promptly identify pathogenic organisms in positive blood cultures and improve patient outcomes.

The FilmArray® Sepsis System includes a real time PCR instrument and novel thin film pouch. Together these perform automated nucleic acid purification and nested multiplex PCR to analyze up to 102 nucleic acid targets from a single sample in ~1 h. The FilmArray Sepsis System tests for >19 sepsis-causing organisms and several antibiotic resistance genes via PCR amplification. Bacteria is identified by housekeeping and virulence gene targets. Antibiotic resistance targets include mecA, vanA-C, ermB and ESBL and carbapenemase resistance genes. PCR primers were designed by aligning bacterial sequences from GenBank. Outer PCR primers were tested for efficiency in both singleplex and multiplex reactions using traditional real time PCR instruments. Inner PCR primers were tested as singleplex reactions only. Primers with high efficiency were transferred to the FilmArray.

Bacterial isolates representing targets on the entire panel were used to validate the panel. Both ATCC strains and clinical isolates have been accurately identified. Antibiotic-resistant strains tested include, MRSA, VRE and a KPC-positive K. pneumoniae.

Preliminary testing with 60 clinical samples from blood culture bottles demonstrated accurate identification of bacteria in 100% (60/60) of positive blood cultures when compared to standard microbiologic testing. Testing of culture-negative samples showed no amplification over background.

The FilmArray Sepsis System is a novel tool for the detection and identification of sepsis-causing pathogens in clinical samples positive blood cultures. Multiplex testing allows each sample to be evaluated for a large number of pathogens simultaneously. Preliminary testing demonstrates the utility of this system in the rapid identification of pathogens from positive blood cultures.

INTRODUCTION

Sepsis is a leading cause of morbidity and mortality in the U.S. and the world. Rapid initiation of appropriate antibiotic therapy is crucial. Current methods of pathogen identification are slow, leading to delays in diagnosis. Blood cultures can take 24-48 hours to become positive. After cultures become positive another 24-48 hours is required for complete identification and susceptibility testing. Molecular diagnostics involving PCR for the detection of multiple pathogens and mechanisms of antibiotic resistance could provide more rapid results.

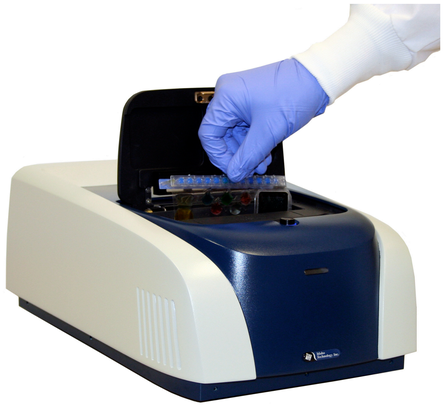
In response to the need for a diagnostic system that can rapidly and easily identify multiple sepsis-causing pathogens from positive blood cultures, ITI is developing the FilmArray® Sepsis System. The FilmArray will have several advantages over conventional culture-based detection. First and foremost, the time to identification of pathogens after a blood culture becomes positive will be significantly decreased, from several days to ~1 hour. Second, the FilmArray provides a low complexity system for the operator, requiring only injection of the blood culture sample into the pouch and starting the instrument.

METHODS

For bacterial and fungal (candida) detection and identification the FilmArray Sepsis System will employ a nested-multiplex PCR strategy described in Figure. 2. Outer primers for housekeeping genes are designed to be broad-range, and are based on distantly related bacterial and fungal species. Gene sequences were obtained through the NCBI database. Inner primers are designed to be species-specific. Assays for virulence targets are made to be species-specific. Antibiotic resistance assays target the gene of interest. Initial testing of FilmArray Sepsis System primer sets was performed on traditional real-time PCR instruments with clinical bacterial isolates from Primary Children's Medical Center (PCMC), Salt Lake City, UT. Successful multiplex assays were transferred to the FilmArray System. Blood culture samples were obtained from PCMC with approval of the Institutional Review Board of the University of Utah and PCMC.

Figure 1: The FilmArray® Instrument and Pouch

ITI has developed a lab-in-a-pouch system called "FilmArray". It is a medium-scale fluid manipulation system performed in a self-contained, disposable, thin-film plastic pouch. The FilmArray platform processes a single sample, from nucleic acid purification to result, in a fully automated fashion. These system characteristics are ideal for the multiplex testing of pathogens in standard diagnostic sample matrices.



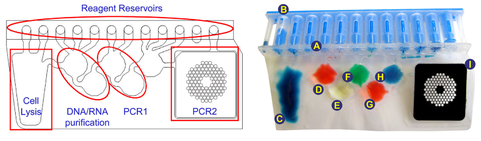
The FilmArray Test System
A FilmArray test is initiated by injecting rehydration solution and a patient blood culture sample into the FilmArray pouch and placing it in the FilmArray instrument. The user enters the sample and pouch type (using a barcode reader) into the software and initiates a run. Results are provided in ~ 1 hour.

The FilmArray pouch has a fitment (see label A) containing all needed freeze-dried reagents.

The film portion of the pouch has stations for:

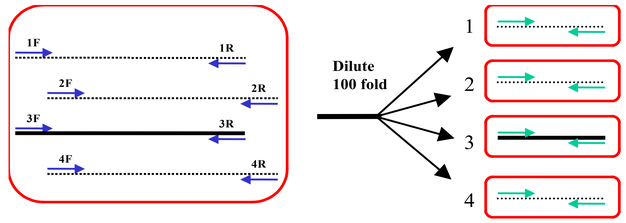
1. Cell lysis (Blister C)
2. Magnetic-bead based nucleic acid purification (D & E)
3. First-stage multiplex PCR (F & G)
4. Array of 102, second-stage nested PCRs (I)

PCR primers are dried into the wells of the array and each primer set amplifies a unique product of the first-stage multiplex PCR. The second stage PCR product is detected in real-time using a fluorescent-double-stranded DNA binding dye, LCGreen®.



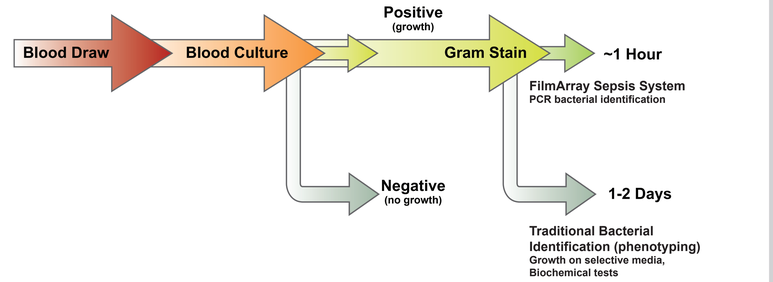
- **A. Fitment with freeze-dried reagents**
- **B. Plungers- deliver reagents to blisters**
- **C. Sample lysis and bead collection**
- **D. Wash station**
- **E. Magnetic bead collection blister**
- **F. Elution Station**
- **G. Multiplex Outer PCR blister**
- **H. Dilution blister**
- **I. Inner Nested PCR array**

Figure 2: Schematic of Nested Multiplex PCR



A large volume multiplex PCR (shown here as 4-plex on the left side of figure) is run for a limited number of cycles (20). The reaction is diluted and distributed to individual small PCR reactions that contain primers (green) nested inside the primers (blue) of the first PCR reaction. A template amplified in the first reaction (by the #3 primers) is further amplified in only one of the second reactions.

Figure 3. Proposed Integration of FilmArray® into Current Blood Culture Procedures



Tables 1 & 2. FilmArray® Sepsis Panel: Pathogens and Targets

1

Gram-positive and Fungi	Gram-negative	Antibiotic resistance targets
Gram-positive	*Escherichia coli*	mecA
Staphylococcus aureus	*Klebsiella oxytoca*	vanA, vanB, vanC
Streptococcus pneumoniae	*Klebsiella pneumoniae*	KPC
Streptococcus agalactiae	*Enterobacter species*	CTX-M
Streptococcus pyogenes	*Neisseria meningitidis*	SHV
Enterococcus species	*Haemophilus influenzae*	TEM
Streptococcus species	*Pseudomonas aeruginosa*	OXA-58
Coagulase negative *staphylococcus*	Enteric species*	ermB
Fungi	*Acinetobacter baumannii*	
Candida albicans	*Serratia marcescens*	
Candida non-albicans	*Bacteroides fragilis*	
	Proteus species	

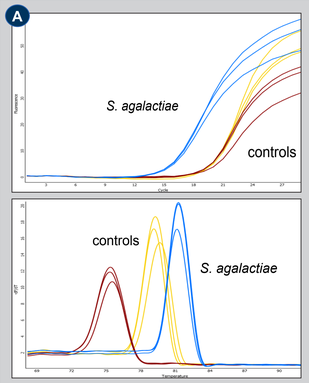
Bacteria in red are still being considered for the final panel
**E. coli, K. pneumoniae, K.oxytoca, Enterobacter* spp, *Citrobacter* spp, *Proteus* spp, *S. marcescens, Salmonella* spp, *Shigella* spp.

2

Housekeeping genes	Virulence factor genes
RNA polymerase B (*rpoB*)	streptococcal pyrogenic exotoxin (*speB*)
gyrase B (*gyrB*)	autolysin (*lytA*)
outer membrane protein A (*ompA*)	capsule export protein A (*ctrA*)
RNA polymerase B, yeast (*RPB1*)	thiouridine sulfurtransferase A (*tusA*)
	nuclease (*nuc*)
	capsule gene (*bexA*)
	surface immungenic protein (*sip*)
	1,3-beta-D-glucan synthase (*FKS1*)

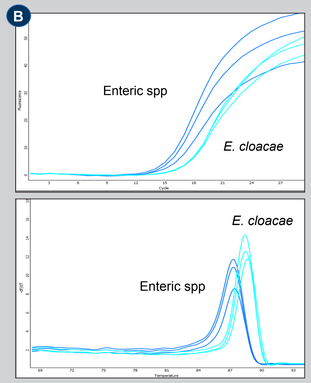
The Sepsis panel identifies bacterial and fungal targets using a combination of highly conserved housekeeping genes and virulence genes. Target genes are carefully chosen through clinical literature research and access to genomic sequencing.

Figure 4. Blood culture detection in the FilmArray® Sepsis Pouch



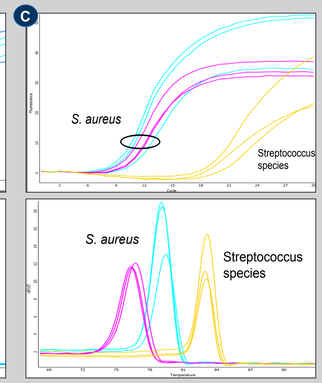
PCR data from a positive blood culture. The sample is diluted 1:100, mixed with a lysis buffer and injected into the pouch. *S. agalactiae* (GBS) is detected by the sip assay. Curves are also shown for the system's internal controls. The sample processing control is an *S. pombe* organism that is freeze-dried into the pouch. This DNA target controls for sample processing, PCR1 and PCR2. The PCR2 specific control is an oligonucleotide spotted onto the array. Both the sample processing control and the PCR2 control must pass for the software to call and positive or negative sample.

Corresponding melt curves for the *S. agalactiae* and control assays.



Blood culture positive for *E. cloacae* injected into the FilmArray Sepsis pouch. *E. cloacae* is identified by the gyrB gene and will also be positive for the enteric spp "pan" assay (ompA gene). Primers for both gene targets are degenerate in the outer. GyrB primers are *E. cloacae* specific in the inner, while ompA primers are still broad in the inner to capture a large range of enteric species.

Melt curves shown for both assays.



Dual detection of S. aureus and *Streptococcus* species in a blood culture sample. Positive blood culture specimen injected into the FilmArray Sepsis pouch shows identification of *S. aureus* by two different assays: rpoB (housekeeping gene; blue), and nuc (virulence gene; pink). Outer primers for rpoB capture a broad spectrum of Gram-positive bacteria and inner primers are specific for *S. aureus*. Corresponding melt peaks for the assays are also shown.

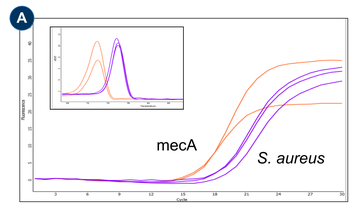
Positive amplification of the *Streptococcus* species assay (rpoB) from the same positive blood culture specimen is shown in yellow. By culture this sample grew both *S. aureus* and *S. mitis*. The FilmArray Sepsis pouch will not report a species for the *S. mitis* organism, but will call "*Streptococcus* species". This assay identifies a variety of viridans streptococci, beta-hemolytic streptococci, and alpha-hemolytic streptococci. Separate assays for *S. pyogenes*, *S. agalatiae*, and *S. pneumoniae* will distinguish these from other *Streptococcus* species. Nested multiplex PCR allows for the identification of two (or more) organisms in one sample.

Corresponding melt peaks for the assays are also shown.

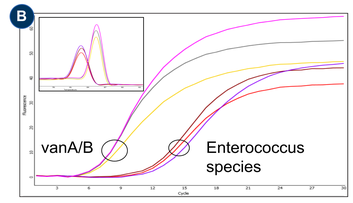
Organism	No. Detected by Blood Culture	No. Detected by the FilmArray
Staphylococcus aureus	10 (2 MRSA)	10* (2 MRSA)
Streptococcus pneumoniae	8	8
Streptococcus agalactiae	4	4*
Streptococcus pyogenes	3	3
Enterococcus species	10 (1 VRE)	10* (1 VRE)
Streptococcus species	5	5
Coagulase negative staphylococcus	7	7*
Escherichia coli	3	3
Klebsiella oxytoca	2	2
Klebsiella pneumoniae	3	3
Enterobacter species	1	1
Neisseria meningitidis	1	1
Haemophilus influenzae	1	1
Pseudomonas aeruginosa	2	2
Acinetobacter baumannii	0	0
Serratia marcescens	1	1

* We believe that several samples were inadvertently interchanged in the aliquoting process. A *S. aureus* positive by culture was positive for *S. agalactiae* on the FilmArray while the tube next to that sample was positive for *S. agalactiae* by culture and *S. aureus* by the FilmArray. We assume the same thing happened with two other samples, this time involving a switch between an *Enteroccocus* species and a CONS.

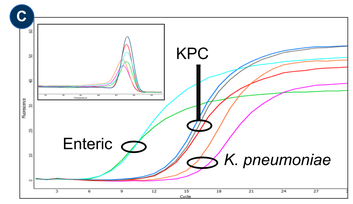
Figure 5. FilmArray® detection of antibiotic resistant organisms MRSA, VRE, and KPC containing *K. pneumoniae.*



Blood culture injected into the FilmArray Sepsis pouch, showing detection of Methicillin resistant *S. aureus* (MRSA). *S. aureus* is identified by the nuc (nuclease) gene in purple. MecA has also been detected in the sample, however the software call is "mecA detected", as mecA is sometimes found in some coagulase-gative staphylococci. Melts for the assays are shown (inset). Conventional susceptibility testing at PCMC determined this isolate was resistant to methicillin.



Enteroccocus faecalis (organism) injected into the pouch is identified by the rpoB target assay. Vancomycin-resistance genes vanA/B are also detected in this organism. Inner primers for vanA and vanB and vanC gene are multiplexed on the inner array. VanA and B amplicons have identical melting curves while VanC is distinguished by a lower Tm. Melt curves for the vanA/B assays and rpoB Enterococcus species assay show differences in amplicon Tm. This allows confirmation of specific amplification (inset). Conventional susceptibility testing at PCMC determined this isolate was resistant to vancomycin.



ATCC *K. pneumoniae* strain (BAA-1705) known to contain the KPC gene injected into the FilmArray Sepsis pouch. *K. pneumoniae* is identified by two housekeeping genes, gyrB and ompA. GyrB has specific inner primers for *K. pneumoniae*. The ompA assay is "pan-enteric" and detects multiple species of enteric organisms. The KPC gene, *K. pneumoniae* carbapenemase, (not limited to *K. pneumoniae*), confers resistance to carbapenem antibiotics. Melt profiles for the three assays are shown (inset).

CONCLUSIONS

1. **Nested multiplex PCR targeting of housekeeping genes and virulence genes accurately identifies bacteria from positive blood cultures.**
2. **Nested multiplex PCR targeting antibiotic resistance genes accurately identifies resistant bacterial isolates.**
3. **The FilmArray® Sepsis System will be an excellent tool for rapid identification of pathogens in positive blood cultures and will decrease the time between a positive culture and full ID.**

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