C-113

Rapid Molecular Diagnostics for Serious Bacterial Infection

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Abstract

Background: Ten percent of infants presenting with fever have a serious bacterial infection (SBI). Expert consensus guidelines recommend a burdensome and expensive management strategy pending bacterial culture results. We are developing rapid diagnostics that will allow prompt identification of infants with SBI, and prevent unnecessary hospitalization and antibiotic treatment of low-risk infants.

Methods: Idaho Technology, Inc (ITI) has developed a portable real-time PCR platform, the FilmArray, that uses nested multiplex PCR (nmPCR) to analyze up to 120 nucleic acid targets in an unprocessed clinical sample, with a time-to-result of 60 minutes. The Febrile Infant Risk Stratification Tool ("FIRST Assay") is under development for the FilmArray platform as a collaboration between the University of Utah and ITI. PCR primers were designed based on alignment of bacterial DNA sequences from GenBank. Clinical bacterial isolates were used in assay development.

Results: The FIRST Assay targets conserved bacterial housekeeping genes, including the RNA polymerase beta subunit (*rpoB*) and the DNA gyrase subunit b (*gyrB*). Degenerate outer primers are broad-range, and amplify targets in diverse bacterial species; inner primers target pathogenic species of interest. Identification of 14 bacterial pathogens, including *E. coli*, *S. agalactiae* and *S. aureus* was tested using this system. With only 4 outer primer pairs, appropriate targets from both *rpoB* and *gyrB* were amplified in all bacteria tested. Inner primers were specific for their target species, and all 14 species were accurately identified as confirmed by sequencing of both genes.

Conclusions: We have shown proof-of-principal for the use of nmPCR targeting multiple conserved housekeeping genes for the accurate identification of bacteria causing infection in febrile infants. Broad-range outer primers provide the potential to easily modify the inner targets for different patient populations. When fully developed for the FilmArray, the FIRST Assay will provide a tool for the rapid and accurate evaluation for SBI in infants with fever.

Introduction

Care of febrile infants younger than 90 days of age is controversial and is a significant problem in the US. Twenty percent of physician visits in this age group are for fever.[3] Approximately 10% of these infants have serious bacterial infections (SBI) including bacteremia and meningitis. However, 90% of febrile infants have self-limited viral infections.

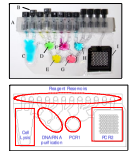
Until recently, rapidly distinguishing infants with viral illness from those with bacterial infection has been impossible. Because of the potentially fatal consequences of delayed treatment of SBI, pediatric guidelines recommend an aggressive evaluation for sepsis, broad-spectrum antibiotic therapy, and hospital admission for the majority of febrile infants.[4] These guidelines result in thousands of 3-day hospital admissions each year in the US. The negative consequences associated with hospitalization of these young infants include adverse drug events, discontinuation of breast-feeding, and unnecessary parental anxiety that may have long-term psychological consequences for the infant. Hospital-acquired infection is an additional risk. Financial costs incurred nationally are significant, with admissions averaging in excess of $5,000 each. Public health costs related to antibiotic resistance resulting from antibiotic treatment of thousands of infants with viral infections have not been calculated.

Current technology for "rapid detection" of viral and bacterial pathogens requires at least 24 hours and multiple tests to exclude the most common pathogens. A rapid test to exclude or confirm bacterial infection, would be invaluable in the care of the febrile infant.

Methods

For bacterial detection and identification the FIRST Assay employs a nested-multiplex PCR strategy described in Figure 2. Outer primers are designed to be broad-range, and are based on alignments of housekeeping gene targets (*rpoB* and *gyrB*) from distantly related bacterial species. Gene sequences were obtained through the NCBI database and aligned based on amino acid sequences to identify protein domains conserved across species. Outer primers target these domains, with degenerate based incorporated in some positions to facilitate cross-species recognition. Inner primers are designed to be species-specific, and are placed in locations where the 3' end includes a characteristic amino acid "signature" conserved among isolates of the same species, but different in other species. Primer 3' mismatches for non-targets help limit cross-amplification. Initial testing of FIRST Assay primer sets was performed on the benchtop with clinical bacterial isolates from Primary Children's Medical Center, Salt Lake City, UT; successful multiplex assays were transferred to the FilmArray platform (FilmArray shown in Figure 1, Data in figures 5 and 6).

Figure 1: The FilmArray Instrument and Pouch



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

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 NPA 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

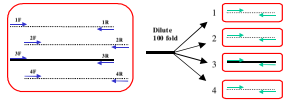
A FilmArray test is initiated by injecting water (to rehydrate reagents) and a patient sample (NPA or NPS) into the FilmArray pouch and placing it in the FilmArray instrument. The user enters the sample and pouch type (using a barcode reader) into the software and initiates a run. Results are provided in less than one hour.

The FilmArray pouch has a fitment (label A) containing all needed freeze-dried reagents. The film portion of the pouch has stations for:

- Cell lysis (Blister C)
- Magnetic-bead based nucleic acid purification (D & E)
- Reverse transcription (RT) to detect RNA viruses (F & G)
- First-stage multiplex PCR (F & G)
- Array of 120, second-stage nested PCRs (I)

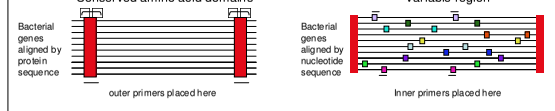
PCR primers are dried into the wells of the array and each primer set amplifies a unique product of the first-stage multiplex PCR. The second stage PCR product is detected in real-time using a fluorescent-double-stranded DNA binding dye, LCGreen Plus, developed by ITI and post-PCR product identity is confirmed by high-resolution melt profiling.

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 real-time 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: Schematic of Nested Primer Design for Bacterial Identification



A. Outer primers are designed to be broad range and amplify multiple bacterial species across phyla. This is accomplished by targeting conserved protein domains (red boxes) in which the amino acid sequence is the same for all bacteria of interest. Degenerate bases are used within primer sequences to account for variability in the coding sequence.

B. Inner primers are designed to be specific for the target species. This is accomplished by designing these primers within variable regions between the conserved domains targeted in the outer PCR. Primers are placed where sequences are the same across those available for a particular bacterial species, but different from that of other species. This can be difficult for closely related organisms (see Figure 6).

Bacterial Targets	
Gram-positive	**Gram-negative**
Staphylococcus aureus	*Escherichia coli*
Staphylococcus epidermidis	*Klebsiella pneumoniae*
Streptococcus agalactiae	*Klebsiella oxytoca*
Streptococcus pneumoniae	*Enterobacter cloacae*
Streptococcus pyogenes	*Haemophilus influenzae*
Listeria monocytogenes	*Neisseria meningitidis*

Housekeeping Gene Targets
rpoB
gyrB
groEL
ompA
16S rRNA gene

Figure 4: Example Algorithm for Management of Febrile Infants

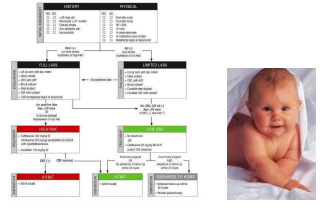
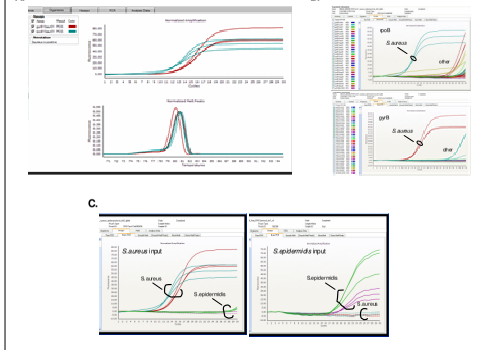


Figure 5: *S.aureus* identification in the FilmArray



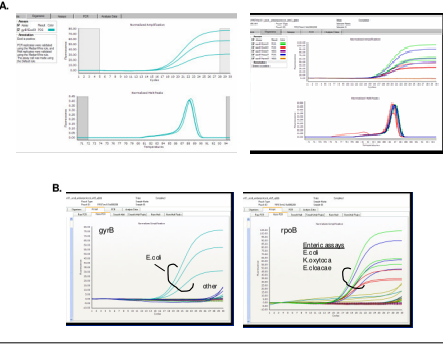
Nucleic acid extracted from a clinical isolate of *S.aureus* was injected into a FilmArray pouch. The outer multiplexPCR contained broad-range primers targeting both the *rpoB* and *gyrB* genes. Inner PCR primers were spotted into the high-density array. Within the inner array are primers targeting 14 different pathogenic organisms.

A. FilmArray analysis software shows identification of *S.aureus* based on real-time amplification curves (top) and DNA melting profile curves (bottom) generated by the *S.aureus*-specific primers for both genes. Only the wells with *S.aureus* primers are shown here.

B. Raw data from the assay run showing all wells demonstrates the specificity of the *S.aureus* primers and preferential amplification of the target organism. Late amplification within other wells is related to primer dimer formation.

C. Raw data showing differentiation of *S.aureus* and *S.epidermidis* by the FIRST Assay. The left-sided panel shows both the *S.aureus* and *S.epidermidis* assays when *S.aureus* is injected into the pouch. On the right, data from a run where *S.epidermidis* nucleic acid was injected.

Figure 6: *E.coli* identification in the FilmArray



Nucleic acid extracted from a clinical isolate of *E.coli* was injected into a FilmArray pouch. The pouch used for this run contains the same broad-range outer and species-specific inner primers as that used for the experiment shown in Figure 4.

A. For enteric organisms such as *E.coli*, the analysis software looks at the *gyrB* and *rpoB* assays separately. *gyrB* assays are species-specific, while *rpoB* assays for each enteric organism (*E.coli*, *K.pneumoniae*, *K.oxytoca* and *E.cloacae*) cross-amplify. The pattern of amplification over over the primer sets, along with the results of the *gyrB* amplification, defines the enteric species.

B. Raw data from the assay run showing all wells demonstrates the specificity of the *gyrB* assay for *E.coli*. *gyrB* assays targeting *K.pneumoniae*, *K.oxytoca* and *E.cloacae* are also included in the array. For *rpoB*, only those assays targeting enteric organisms show significant amplification. The *rpoB K.pneumoniae* assay failed in this set of pouches

Conclusions

- These data show proof-of-principal for the use of nmPCR targeting conserved housekeeping genes for the accurate identification of bacteria
- Bacteria that can be identified in the FIRST Assay are those causing infection in febrile infants. These bacteria can be differentiated from skin contaminants such as *S.epidermidis*.
- Broad-range outer primers provide the potential to easily modify the inner targets for different patient populations.
- When fully developed for the FilmArray, the FIRST Assay will provide a tool for the rapid and accurate evaluation of serious bacterial infection in infants with fever.