Friday 434

Development of a Multiplex Molecular Assay for the Rapid Detection of Unrecognized Bacteria Causing Pharyngitis

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Abstract

Background: *Fusobacterium necrophorum, Arcanobacterium haemolyticum,* and *Streptococcus dysgalactiae* are often unrecognized and potentially serious causes of pharyngeal disease. Routine bacterial isolation and identification of these organisms is hindered due to their fastidious nature, requiring special media and incubation conditions. Furthermore, there are currently no commercially available tests for detection of these pathogens. A rapid, sensitive, and specific multiplex assay would improve differential diagnosis for pharyngitis, allowing for more focused antimicrobial therapy.

Methods: A Target-Enriched Multiplex PCR (TEM-PCR™) Bacterial Pneumonia Panel (BPP) assay was redesigned to include targets specific for *F. necrophorum, A. haemolyticum,* and *S. dysgalactiae,* along with 16 other bacterial targets. DNA extraction was performed using stock organisms of known titer followed by TEM-PCR with subsequent detection on low density microarrays. Specificity of the assays was tested both *in silico* and *in vitro* with phylogenetically related organisms, normal flora, or pathogenic organisms that may be present at the sampling site. Additionally, 510 clinical samples from 15-30 year olds with presentation of pharyngitis symptoms were tested to determine preliminary prevalence rates for these three pathogens.

Results: The analytical limit of detection was calculated for each target resulting in a LoD^{95} of 135 GEq/mL for *F. necrophorum,* 9120 cfu/mL for *A. haemolyticum,* and 5.0e+04 cfu/mL for *S. dysgalactiae.* Off-target bacteria and viruses (n=102) were tested at 1e6 cfu/mL and 1e4 pfu/mL, respectively, with no detected cross-reactivity to any assay present in the BPP Panel. Inclusivity of the assay was demonstrated using various strains (n=43) expected to have coverage of the target genes. The assay designed for detection of *F. necrophorum* was also capable of differentiating between *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme.* Preliminary prevalence rates in clinical samples were 15/510 (2.7%) for *F. necrophorum*, 4/510 (0.7%) for *A. haemolyticum*, and 18/510 (3.3%) for *S. dysgalactiae*.

Conclusion: The TEM-PCR BPP assay is the first multiplexed PCR test available for *F. necrophorum, A. haemolyticum,* and *S. dysgalactiae*, allowing for accurate and sensitive detection of under-reported organisms causing pharyngitis. The BPP assay is performed directly from a patient specimen and can detect multiple pathogens in a single specimen in less than eight hours, eliminating empiric antibiotic therapy and resulting in faster, more accurate patient treatment.

Note: Abstract amended to include additional test sample data

Introduction

- *Streptococcus pyogenes* (Group A Strep) is often the only potential causative agent considered when a patient presents with symptoms of pharyngitis.
- *A. haemolyticum, F. necrophorum,* and *S. dysgalactiae* often go undiagnosed as causes of pharyngitis due to use of Group A Strep rapid ID tests and fastidious nature of the organisms.
- *F. necrophorum* is a primary cause of Lemierre's Syndrome, a potentially life-threatening septicemic disease in adolescents, making accurate and prompt diagnosis critical.[1]
- *A. haemolyticum* has been implicated as a cause of pharyngitis and is frequently isolated in association with other organisms which makes its pathogenic significance a challenge to determine.[2]
- Beta-hemolytic *S. dysgalactiae* is the causative agent of Group C/G streptococcal infection and is often missed due to the similarity of its clinical presentation to *Streptococcus pyogenes.* Recent studies suggest prevalence rates could be approaching 5%.[3]
- There are currently no commercially available multiplex molecular tests for *A. haemolyticum, F. necrophorum,* and *S. dysgalactiae*, and no commercially available molecular tests of any kind for *A. haemolyticum* or *F. necrophorum.*
- A rapid and sensitive multiplex assay would greatly improve pharyngitis diagnoses and allow for a more specific approach to appropriate antibiotic treatment.

Materials & Methods

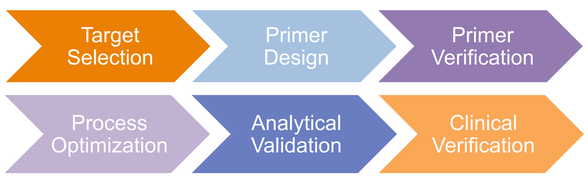


Figure 1. TEM-PCR Bacterial Pneumonia Panel Development Workflow.
Summary of processes involved in the creation of the BPP panel.

Panel Development. Development of the Bacterial Pneumonia Panel (BPP) went through multiple design and verification processes, per Clinical and Laboratory Standards Institute guidelines[4]. These steps help to ensure optimal performance of TEM-PCR panels in terms of sensitivity and specificity. Extraction, TEM-PCR setup, and detection procedures are all optimized for assay function.

Materials & Methods *(continued)*

Assay Design and Verification. Gene targets for organisms in the BPP assay were selected based on literature research and clinical relevance. Nested primers were designed based on sequence information available in the National Center for Biotechnology Information (NCBI) database. The assay specificity was verified by real-time PCR using SYBR chemistry. PCR analyses were performed on the CFX96 cycler (BioRad, Hercules, CA).

Table 1. Bacterial Pneumonia Panel Targets.

Acinetobacter baumannii	*Neisseria meningitidis*
Arcanobacterium haemolyticum	*Pseudomonas aeruginosa*
Chlamydophila pneumoniae	*Bordetella pertussis*
Fusobacterium necrophorum	*Staphylococcus aureus*
Haemophilus influenzae type B	*Streptococcus pneumoniae*
Haemophilus influenzae types A-F	*Streptococcus pyogenes*
Klebsiella pneumoniae	*Streptococcus dysgalactiae*
Mycoplasma pneumoniae	Panton-Valentine Leukocidin (PVL) gene
Legionella pneumophila	Methicillin Resistance (Staphylococcal)
Moraxella catarrhalis	

Nucleic Acid Extraction. Extractions were performed on the KingFisher™ Flex (Thermo Fisher Scientific, Waltham, MA) using in-house methods with reagents from Omega Bio-tek (Norcross, GA) and MO BIO Laboratories (Carlsbad, CA).

Target Enriched Multiplex Polymerase Chain Reaction (TEM-PCR) Setup. DNA extracted from serially diluted bacteria was amplified with the BPP assay using TEM-PCR.

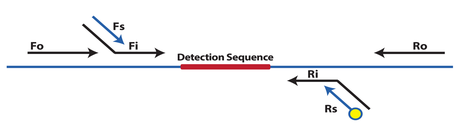


Figure 2. TEM-PCR Scheme.
Low concentration nested gene-specific primers (Fo – forward out; Fi – forward in; Ri – reverse in; and Ro – reverse out) are designed to enrich the targets during the initial PCR cycles. Later in the procedure, a pair of universal SuperPrimers (Fs and Rs) is used to amplify all targets. The Rs primer is labeled with biotin for subsequent detection.

Hybridization and Detection. TEM-PCR amplicons were hybridized to target detection probes on custom microarray plates (Microarrays, Inc., Huntsville, AL). A streptavidin-phycoerythrin solution (Moss, Inc., Pasadena, MD) was added to the hybridization reaction, followed by a series of wash steps. Results for each target were determined to be "Detected" or "Not Detected" based on signal intensity above measured background.

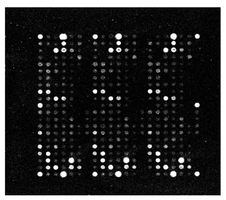
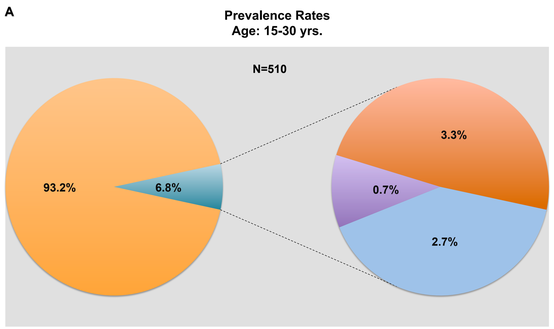


Figure 3. Microarray Plate layout.
Fluorescent signals were analyzed on a SensoSpot® Fluorescence Microarray Reader (Sensovation, Radolfzell, Germany). Arrays are aligned by using a series of reference markers to properly grid each sample well. Target detection probes are spotted in triplicate on the glass surface. Software analyzes the fluorescent intensity of each detection probe. Relative Fluorescent Units (RFUs) are calculated from the average intensity of the three target detection probes.

Analytical Validation. Validation of the BPP assay was carried out over multiple days, using multiple lots of reagents, and multiple lab technicians. Testing was performed to determine sensitivity, inclusivity, exclusivity, and reproducibility of the BPP assay per CLSI guidelines[4].

Clinical Sample Collection. Prevalence rates for *A. haemolyticum, F. necrophorum,* and *S. dysgalactiae* were determined using clinical patient specimens (n=2344), presenting with symptoms of pharyngitis. Samples were collected from the Diatherix clinical laboratory and tested using the BPP assay. Results for 15-30 year old patients (n=510) were analyzed independently from the remaining samples (n=1834) in order to examine the potential increased prevalence of these organisms in young adults. Prior studies have shown *F. necrophorum* to be particularly prevalent in young adults[5]. Co-detection rates with other targets in the BPP assay were determined for *A. haemolyticum, F. necrophorum, S. dysgalactiae,* and *S. pyogenes.* Co-detection rates for *S. pyogenes* were used as a standard for comparison with the three under reported pathogens.

Results



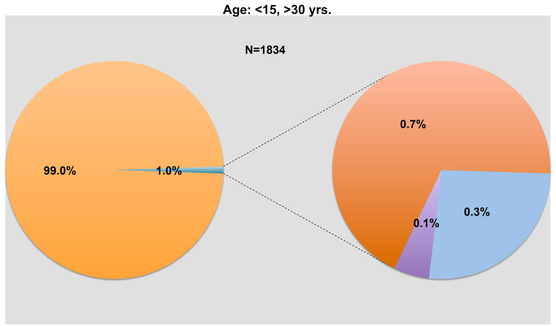


Figure 4. Prevalence Data for *A. haemolyticum, F. necrophorum,* and *S. dysgalactiae* in Different Age Groups. Percent positive for 15-30 year olds (n=510) **(A)** and all other ages (n=1834) **(B)** in clinical samples. Prevalence rates between the two age groups were statistically significant for *F. necrophorum* (p=0.0001), *A. haemolyticum* (p=0.0228), and *S. dysgalactiae* (p=0.0001). "Other" represents clinical samples which were not positive for one of the three under-reported pathogens in this study.

Table 2. BPP Limits of Detection.
LoD^{95} for each target in the BPP assay was determined using the Reed-Muench method[6]. Genome equivalents were calculated after extraction of DNA for *F. necrophorum*. All targets were tested at 10x, 1x, 0.1x LOD.

Target Organism	LoD^{95}	Units
Acinetobacter baumannii	8318	cfu/mL
Arcanobacterium haemolyticum	9120	cfu/mL
Chlamydophila pneumoniae	<50	ifu/mL
Fusobacterium necrophorum	135	GEq/mL
Haemophilus influenzae (HFLUB)	87	cfu/mL
Haemophilus influenzae (HFLU5)	871	cfu/mL
Klebsiella pneumoniae	955	cfu/mL
Legionella pneumophila	8710	cfu/mL
Moraxella catarrhalis	8128	cfu/mL
Mycoplasma pneumoniae	75	cfu/mL
Neisseria meningitidis	78	cfu/mL
Pseudomonas aeruginosa	76	cfu/mL
Bordetella pertussis	8913	cfu/mL
Streptococcus dysgalactiae	50,000	cfu/mL
Streptococcus pneumoniae	38	cfu/mL
Streptococcus pyogenes	4677	cfu/mL
Staphylococcus aureus (mecA)	8318	cfu/mL
Staphylococcus aureus (nuc)	7943	cfu/mL
Staphylococcus aureus (PVL)	8128	cfu/mL

Results *(continued)*

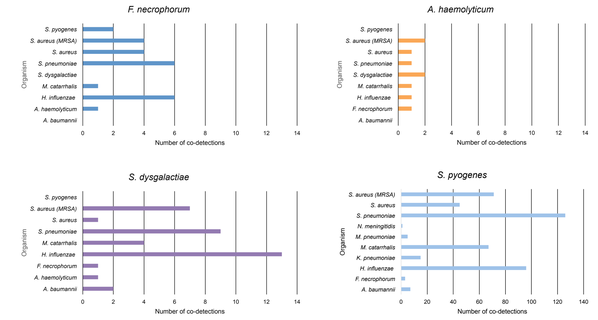


Figure 3. Co-detection Rate of *A. haemolyticum, F. necrophorum,* and *S. dysgalactiae* With Other Respiratory Pathogens in Clinical Specimens. Total number of all tested samples (n=2344); positive for *A. haemolyticum* (n=6), *F. necrophorum* (n=20), *S. dysgalactiae* (n=30), and *S. pyogenes* (n=250). Scaling for *S. pyogenes* is different due to the higher number of positive results.

Conclusions

- The Bacterial Pneumonia Panel assay detected a total of 7% (36/510) of potentially unreported pathogens in young adult (15-30 year-old) clinical samples. Only 1% (20/1834) of other age groups were positive for these same pathogens.
- This difference in prevalence rates between the two age groups is statistically significant and is expected for *F. necrophorum,* as it is more likely to be a causative agent of disease in young adults[1].
- Prevalence rates reported in this study for *A. haemolyticum* and *S. dysgalactiae* are concordant to current reported rates, while the detection rate in this study for *F. necrophorum* is lower than previously reported[5].
- The Bacterial Pneumonia Panel assay has 100% specificity when testing cross-reactivity (n=102) and inclusivity (n=43) strains. The *F. necrophorum* target is specific for only the human strain, subspecies *funduliforme*.
- *Haemophilus influenzae* (35.7%), *Streptococcus pneumoniae* (28.6%), and Methicillin Resistant *Staphylococcus aureus* (23.2%) were the most frequently co-detected organisms observed with *A. haemolyticum, F. necrophorum*, and *S. dysgalactiae* (n=56). These three organisms were also the most commonly co-detected organisms with *S. pyogenes* (n=250), 38.4%, 50.4%, and 28.4%, respectively.
- The Bacterial Pneumonia Panel assay is performed directly from a patient specimen and can detect multiple pathogens in less than eight hours.
- The Diatherix Bacterial Pneumonia Panel assay is the first multiplexed PCR test for accurate and sensitive detection of potentially under-reported organisms causing pharyngitis, as well as the primary causative agent of pharyngitis, *S. pyogenes*.
- Proper and timely detection of *A. haemolyticum, F. necrophorum,* and *S. dysgalactiae* can lead to appropriate administration of antibiotic therapies and improve patient outcomes.

Acknowledgements
We would like to express thanks to Dr. Robert M. Centor for his insights into clinical relevance of these under-reported pathogens.

References
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