

Utility of a Multiplex PCR Panel for the Detection of Bacterial and Viral Targets in Respiratory Secretions

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Abstract

Background: Existing methods that are used in the identification of respiratory infections such as culture, lateral flow immunoassay, and conventional PCR have a limited spectrum of detection. The poly-microbial nature of respiratory infections often involve bacterial and viral pathogens that produce complex pneumonias with a protracted clinical course and poor patient outcomes. This study shows the benefit of a true multiplex technology that can detect both viral and bacterial pathogens in a single specimen.

Methods: Target Enriched Multiplex Polymerase Chain Reaction (TEM-PCRTM) technology was used in this study and incorporates a set of nested primers for the target enrichment step and biotinylated tagged primers for the amplification step. The PCR products that were produced in a strictly controlled amplification environment were detected on a microarray platform with target-matched detection oligonucleotides attached. Results were reported as 'detected' when fluorescence values were above a predetermined cutoff that were specific for each target. Analytical validation of the panel included accuracy, reproducibility and sensitivity steps. The limits of detection (LoD95) were determined using serial dilutions of organisms followed by data analysis with the Reed-Muench method. Data mining of clinical samples collected during the 2012-2013 flu season focused on prevalence rates of co-detection with bacterial targets.

Results: Analytical validation showed the LoD95 for viral targets ranged from 1 pfu/mL (H1N1-09, H3N2, RSV, Coronavirus) to 1000 pfu/mL. The LoD95 for the bacterial targets were below 1000 cfu/mL for most targets and ranged from < 80 cfu/mL (*N. meningitidis*, *S. pneumoniae*) to 7.5e4 cfu/mL. Data mining for the 2012-2013 flu season revealed that the detection rate for the Influenza A target (n=31,320) was 9.92%, comparable to the 9.3% reported by the CDC. Of those specimens containing influenza A, 25% had co-detections with either *S. pneumoniae* (11%), *H. influenzae* (6.8%), or *M. catarrhalis* (8.7%).

Conclusion: The development of a PCR-based, multiplex technology for the detection of bacterial and viral targets in a single specimen is a valuable tool for the diagnosis of respiratory infection; particularly in patients with underlying co-morbidities and risk of developing complex pneumonia.

Introduction

Co-infections with multiple viral pathogens and/or bacterial pathogens have been associated with a protracted clinical course, poor patient outcomes, and increased length of stay when hospitalization is needed. Co-infection in infants with rhinovirus and respiratory syncytial virus has been shown to increase the length of hospitalization by 4.5 days (1). It has been shown that infection with rhinovirus reduces the healing capacity of bronchial epithelial cells and lowers the immune response (2). In cases where the respiratory tract is asymptotically colonized with a potential pathogen, infection with another pathogen can upset the equilibrium resulting in a more severe course of illness as the colonizing pathogen becomes pathogenic. Infection with one microorganism may predispose the individual to additional infection (3). This interplay between the flora of the respiratory tract and the associated potentially worsening effect requires a diagnostic technique that encompasses all of the commonly observed microbial causes of respiratory illness. Data presented here will show the degree of co-infection or co-colonization seen in individuals presenting with respiratory complaints.

Materials & Methods

Sample Collection

- Nasopharyngeal aspirates or swabs were collected from symptomatic patients by physicians distributed throughout the United States and transported overnight to Diatherix Laboratories (Huntsville, AL) in Copan ESwabTM (Murrieta, CA) liquid transport media tubes.
- Specimens must have been no more than 5 days old to be accepted for testing.

DNA extraction

- Specimens were split and aliquots extracted using the Omega Bio-Tek, Inc. Mag-Bind[®] Bacterial DNA 96 Kit (Norcross, GA) and a custom viral kit from MO BIO Laboratories, Inc. (Carlsbad, CA) on a ThermoFisher Scientific, Inc. KingFisherTM Flex semi-automated platform.

PCR and Detection

Extracted DNA and RNA samples were amplified using the target enriched multiplex PCR (TEM-PCRTM) Respiratory Infection Panel. TEM-PCRTM is a highly multiplexed, nested, end-point PCR technique covered by US patent 7,851,148 B2. The Respiratory Infection Panel contains 185 target specific primers which can identify 36 different respiratory pathogens. The end-point PCR products generated by TEM-PCRTM were detected on a custom microarray (Microarrays, Inc., Huntsville, AL).



Figure 1. TEM-PCRTM Scheme. Low concentrations of nested gene-specific primers (Fo – forward out; Fi – forward in; Ri – reverse in; and Ro – reverse out) are designed to enrich the genetic targets during the initial PCR cycles. Later in the procedure, a pair of universal SuperPrimers (FS and RS) are used to amplify all targets. The reverse SuperPrimer is labeled with biotin. Target-specific biotinylated PCR products are detected with a complimentary detection probe which is covalently coupled to a glass microarray.

Results

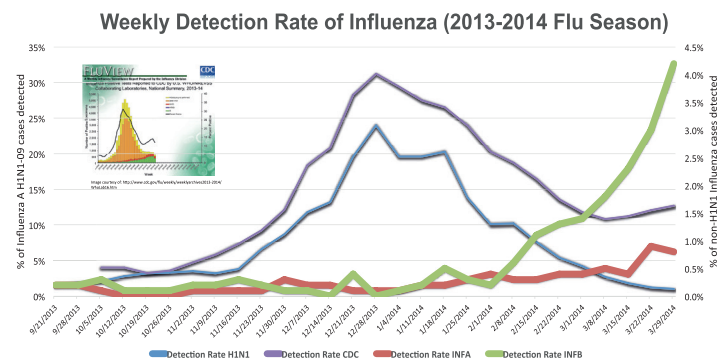


Figure 2. Predominance of Influenza A H1N1 During the 2013-2014 Flu Season (n=22,809) Weekly detection rates of influenza indicated that the Influenza A H1N1-09 was the predominate flu strain. During the 2009 Influenza H1N1 pandemic, secondary bacterial infections in addition to H1N1 infection resulted in increased mortality and severity of illness. As the 2013-2014 flu season has demonstrated, the H1N1 strain is still present and relevant underscoring the need to remain vigilant to the spectrum of pathogens and not simply rely on a one organism test such as the rapid flu tests.

TEM-PCRTM Respiratory Infection Panel

Adenovirus types 3, 4, 7, 21	Acinetobacter baumannii
Coxsackievirus/ Echovirus	Bordetella pertussis
Human bocavirus	Chlamydia pneumoniae
Human coronavirus (229E, OC43, NL63, HKU1)	Haemophilus influenzae
Human metapneumovirus	Haemophilus influenzae (Type B)
Influenza A	Klebsiella pneumoniae
Influenza A- H1N1-09	Legionella pneumoniae
Influenza B	Staphylococcus aureus
Parainfluenza virus 1, 2, 3, 4	MRSA- Meth. Res. S. aureus
Respiratory syncytial virus A, B	Moraxella catarrhalis
Rhinovirus	Mycoplasma pneumoniae
Aminoglycoside resistance	Neisseria meningitidis
Cephalosporin resistance (Staphylococcal)	Pseudomonas aeruginosa
Erythromycin/ Clindamycin resistance	Streptococcus pneumoniae
Tetracycline resistance	Streptococcus pyogenes
	Panton-Valentine Leukocidin toxin

Single Pathogen Two Pathogens Three Pathogens Four Pathogens

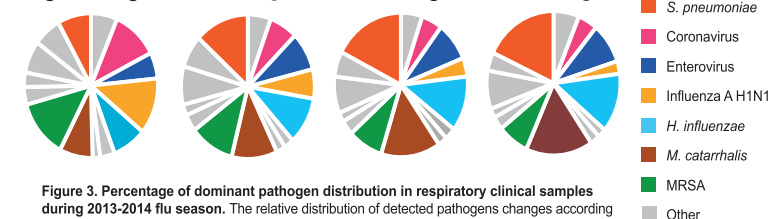


Figure 3. Percentage of dominant pathogen distribution in respiratory clinical samples during 2013-2014 flu season. The relative distribution of detected pathogens changes according to the number of pathogens detected. The percentage of samples where Influenza A H1N1-09 and/or Coronavirus were detected is large when they are the sole organisms detected, but the percentages of H1N1-09 or Coronavirus decline as the number of co-detected pathogen increases with the predominance of viruses being replaced by bacteria. *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are the predominant bacterial strains in clinical respiratory samples.

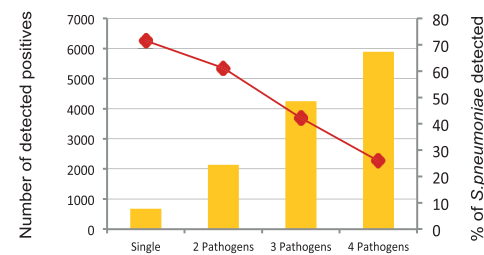


Figure 4. Significant increase in *Streptococcus pneumoniae* co-detection in samples with two or more detected respiratory pathogens.

Results (continued)

Table 1. Co-detection among common viruses and bacteria

	Rhinovirus		Coxsackievirus/Echovirus		Coronavirus	
	Without co-detection No.	With co-detection No. (% of total RHV)	Without co-detection No.	With co-detection No. (% of total CVE)	Without co-detection No.	With co-detection No. (% of total CoV)
<i>S. pneumoniae</i>	2273	1286 (36)	2189	1416 (39)	1784	830 (32)
<i>M. catarrhalis</i>	2261	1308 (37)	2183	1416 (39)	1796	818 (23)
<i>H. influenzae</i>	2548	1021 (29)	2675	924 (26)	2012	602 (23)
Rhinovirus	N/A	N/A	2013	1586 (44)	2345	269 (10)
Coxsackievirus/Echovirus	1983	1586 (44)	N/A	N/A	2360	254 (10)
Coronaviruses	3031	538 (15)	3345	254 (7)	N/A	N/A
Influenza A H1N1	3464	105 (3)	3506	93 (3)	2512	102 (4)
Parainfluenza	3449	120 (3)	3486	133 (4)	2554	60 (2)

Data analysis of three common viruses with respect to their co-detection along with three of the most common bacterial pathogens detected and three more common respiratory viruses did not reveal a trend where infection with one agent predisposes you to co-infection with a second agent in this data set. A large percentage (44%) of patients with rhinovirus were co-infected with either coxsackievirus or echovirus. This association has previously been observed by van den Bergh, et al (3).

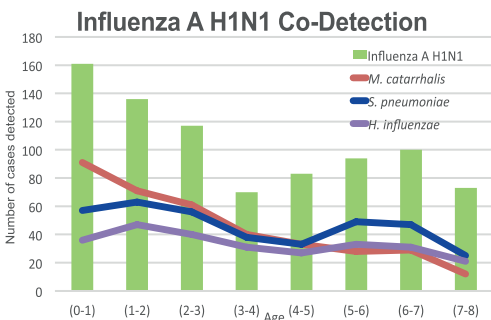


Figure 5. Prevalence of co-detections by pediatric age groups for Influenza A H1N1 infection. *Moraxella catarrhalis*, *Streptococcus pneumoniae*, or *Haemophilus influenzae* represented the three most common bacteria detected in clinical respiratory samples. A bimodality is observed with H1N1 infection that is also observed with the co-detection of *M. catarrhalis*, *S. pneumoniae*, and *H. influenzae*. It is unknown why there is an upward trend in infection with the 4-7 age group. A trend is subsequently observed where the predominate bacterium detected switches from *M. catarrhalis* to *S. pneumoniae* at approximately age 3.

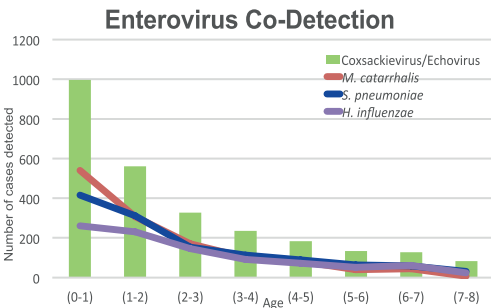


Figure 6. Prevalence of co-detections by pediatric age groups during Enterovirus infection. The age related trend is subsequently observed where the predominate bacterium detected switches from *M. catarrhalis* at approximately age 3 to *S. pneumoniae*. The bimodality is not observed with Enterovirus infection.

Discussion

- Traditional microbiology relies on the skill of the microbiologist to determine the causative agent of infection and typically does not include virology. The source of the infection can be hidden in the cell overgrowth or can fail to grow on the media.
- Single or low-plex viral immunoassays rely on presumptions to determine what organisms to test for. Multiplexed molecular testing gives a complete picture of the spectrum of infection in the patient.
- The 2009 Influenza A H1N1 pandemic demonstrated that bacterial co-infections contributed to a significant worsening of symptoms. The 2013-2014 flu season was predominated by H1N1-09 infection underscoring the need to identify these co-infections.
- Due to effects on the host immune system during a viral infection, it is estimated that 90% of bacterial pneumonias occur due to viral infection (4).
- A complete microbiological picture of the respiratory environment is important to consider and its role in protracted clinical courses of illness. This complete picture can only be gathered from multiplexed molecular testing.
- An interesting trend was observed where patients with one organism detected typically had a viral infection. Where multiple organisms were detected, bacteria predominated. This potentially represents two patient populations, one with acute viral infection and the other with post-viral secondary bacterial infection.

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