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DEVELOPMENT AND VALIDATION OF A QUANTITATIVE MULTIPLEX REAL TIME PCR ASSAY FOR IDENTIFICATION OF BACTERIAL PATHOGENS FROM RESPIRATORY SPECIMENS

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ABSTRACT

Development of timely and accurate methods for diagnosing bacterial respiratory tract infections has been a challenge for the clinical microbiology laboratory. Currently available commercial respiratory panels do not identify some important bacterial pathogens responsible for lower respiratory tract infections (LRTI). Moreover, in many instances, an accurate diagnosis cannot be made due to the overlapping clinical features of bacterial and viral infections on presentation. Culture methods are currently the gold standard for identification of LRTI pathogens. Molecular detection methodologies can offer more sensitive, rapid and targeted identification of these respiratory bacterial pathogens. At the lab, we have validated a laboratory developed sample to answer multiplex PCR for rapid detection of Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis on the Luminex ARIES platform. The assay was evaluated for its analytical performance characteristics and its utility for patient testing in a reference laboratory. In order to provide a comprehensive molecular testing menu for detection of bacterial pathogens, the Aries Group A Strep IVD Assay, a real time PCR assay for detection of Group A Streptococcus pyogenes was also evaluated for its performance characteristics.

KEYWORDS: LRTI, PCR, SPC.

INTRODUCTION

Development of timely and accurate methods for diagnosing bacterial respiratory tract infections have been a challenge for the clinical microbiology laboratory.[1] *Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis* are among some of the major bacterial causes of lower respiratory tract infections (LRTIs) leading to global morbidity and mortality.[2] Currently available commercial respiratory panels do not identify some important bacterial pathogens responsible for lower respiratory tract infections (LRTI). Moreover, in many instances, an accurate diagnosis cannot be made due to the overlapping clinical features of bacterial and viral infections on presentation. Culture methods are currently the gold standard for identification of LRTI pathogens, however this method does have some important drawbacks, such as a long wait period for a result, strict specimen collection and transport protocol and the risk of inhibited pathogen growth due to treatment with prior antibiotics.[3,4] Molecular detection methodologies can offer more sensitive, rapid and targeted identification and diagnosis of these respiratory bacterial pathogens as outlined in the disease management guidelines by the European Respiratory Society.[5] At the lab, we have validated a laboratory developed sample to answer multiplex PCR for rapid detection of *Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis* on the Luminex ARIES platform. The assay was evaluated for its analytical performance characteristics and its utility for patient testing in a reference laboratory. In order to provide a comprehensive molecular testing menu for detection of bacterial pathogens, the Aries Group A *Strep IVD Assay*, a real time PCR assay for detection of Group A *Streptococcus pyogenes* was also evaluated for its performance characteristics.

METHODOLOGY

Pure cultures of bacterial isolates obtained commercially through American Type Culture Collection (ATCC) were used for assay validation. Serial dilutions were prepared for molecular quantification and quantitative comparison with culture. Clinical specimens used for validation were either obtained from the microbiology department at the lab or through specimen exchange with an external laboratory. The assay target genes were chosen from previously published literature; Autolysin

(lyt A) gene for *Streptococcus pneumoniae*, L-Fuculokinase (*fuc K*) gene for *Haemophilus influenzae* and an Outer membrane protein B2 (*cop B*) gene for *Moraxellacatarrhalis*. An internal control target (ssRNA) for Monkey hepatitis virus was included in the assay. The primers and probes were manufactured by IDT. They were placed as a cocktail into the Aries EXO + Ready mix tubes which were then attached to the extraction cassette to perform the real time PCR. Nasopharyngeal swabs were pretreated with Proteinase K whereas sputum samples were pretreated with sputum liquefying reagent Snotbuster [Copan Italia] and Proteinase K prior to extraction of nucleic acid and real time PCR on the ARIES platform. Throat swab samples collected in modified Liquid Amies Medium [COPAN ESwabs] are directly added to Abbott-ID Group A Strep Assay cassette which is placed on Abbott-ID for real time PCR. A sample processing control (SPC) (part of the cassette) is monitored along with Group A Strep. Total assay time including extraction and PCR cycling is approximately 2 hours.

Figures and Tables

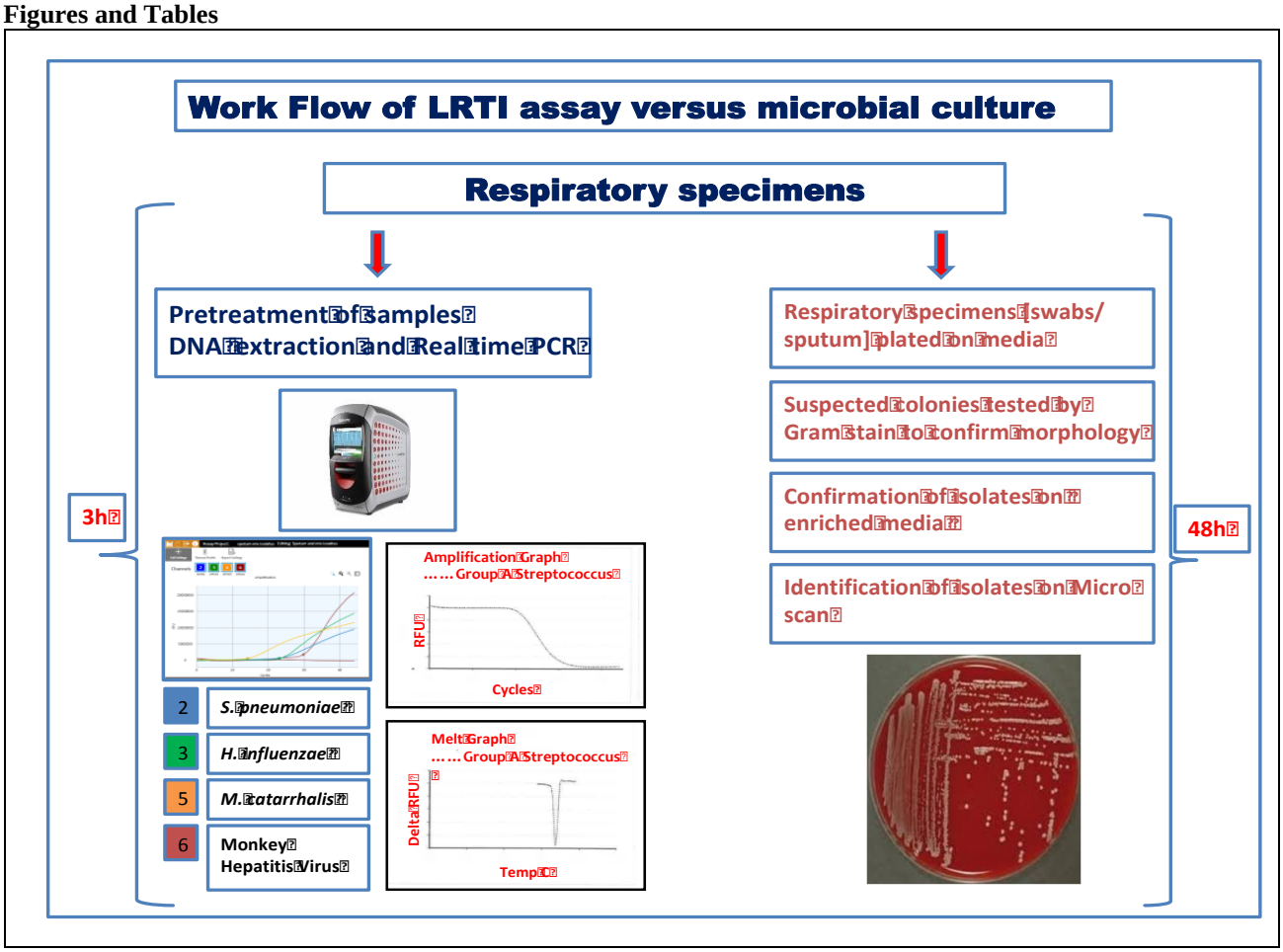

Figure 1 (above): Workflow of the LRTI array versus routine culture methods. The assay involves pretreatment of sample followed by DNA extraction and real time PCR from respiratory specimens.

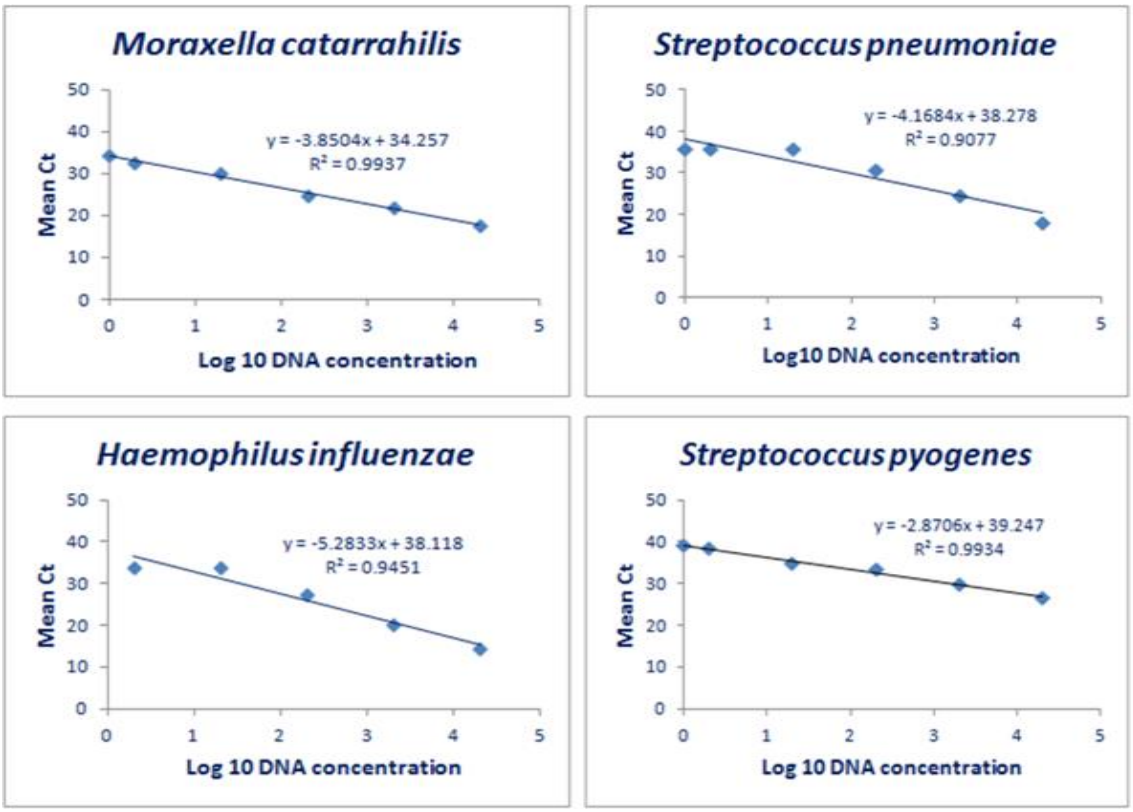

Figure 2 (above): Representation of PCR linearity and quantitative range; Overnight grown cultures of pure control isolates were used to make serial dilutions (six replicates for each concentration) for determination of PCR efficiency, linearity and quantitative range.

A	Summary of Accuracy			
		Aries Real Time PCR (n=35)		
		POSITIVE	NEGATIVE	TOTAL
PCR/ culture	POSITIVE	20	0	20
	NEGATIVE	1	18	19
	TOTAL	40	18	39

Fig A: Summary of accuracy upon testing of clinical specimens. The specimens (n=35) showed 97% concordance between our assays and either culture or molecular testing performed at external laboratories.

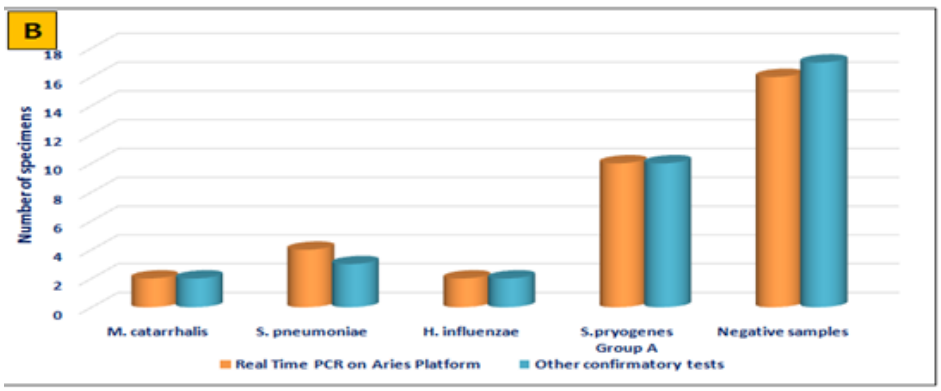

Fig B: Graphical representation of clinical specimen correlation.

Table 2: Determination of a quantitative range for detection of respiratory pathogens using the LRTI assay and its comparison with colony forming units obtained by culture. The molecular multiplex LRTI assay reliably quantified the bacterial pathogens down to a concentration of 150-200 CFU's/reaction.

Moraxella catarrhalis		*Streptococcus pneumoniae*		*Haemophillus influenzae*	
cfu/ml	Mean Ct	cfu/ml	Mean Ct	cfu/ml	Mean Ct
>100000	18.7	>100000	19.1	>100000	15.3
100,000	22.8	100000	25.8	100000	21.3
40-50,000	25.8	50-80,000	31.9	40-50,000	28.3
<10,000	31.0	<10,000	36.9	<10,000	34.8
No growth	33.5	No growth	37.0	No growth	35.0
No growth	35.5	No growth	ND	No growth	35.0

Table 3: Limit of Detection; The LRTI assay was used to determine analytical sensitivity when sputum specimens were spiked with 10ul (corresponding to 800 CFU/ rxn) of each organism equally in a mixture. All the pathogens were reliably identified in 3 replicates.

Isolates spiked in sputum [80,000-100,000 cfu/ml]	Mean Ct observed (corresponding to 800 CFU/rxn)
H. influenzae	29.5
S. pneumoniae	28.0
M. catarrhalis	27.4

RESULTS

- The LRTI multiplex assay is linear over a broad dynamic range of DNA concentrations ranging from 0.3-4.5 log10ng/ml (Fig 2).
- Table 2 depicts the mean assay Ct's obtained when serial dilutions of the isolates were assayed using the LRTI assay and simultaneously plated for determination of CFU's.
- The molecular multiplex LRTI assay reliably quantified the bacterial pathogens down to a concentration of 150-200 CFU's/reaction.
- Accuracy determination was performed using 35 clinical specimens (sputum, throat or nasopharyngeal swabs). The assay was 97% concordant with routine culture or another PCR method. One discordant specimen resulted as positive for S. pneumoniae could not be recovered by routine culture. (Table 1A and Fig B).
- To determine the limit of detection, a triple mixture of isolates was prepared in equal amounts and spiked into sputum. At least 3 replicates were assayed and the assay reliably quantified up to 800 CFU/reaction of all three pathogens in the mixture (Table 3).
- The LRTI assay showed specificity in pathogen detection. All negative clinical specimens were detected as negatives and there was no non-specificity or cross reactivity observed in detection of isolates even with high bacterial loads.

CONCLUSION

The molecular multiplex LRTI assay is a valuable alternative to the traditional culture methods for identification of lower respiratory bacterial pathogens. The LRTI pathogen panel is fast, accurate and highly sensitive which helps in reducing the turnaround time in comparison to culturing pathogens, also known as the gold method, which could take up to 48 hours. Since the LRTI assay identifies the common lower respiratory tract bacterial pathogens responsible for community acquired pneumonia, the assay can be a valuable addition to multiplex viral pathogen panels and therefore this method supports antibiotic stewardship.[6]

KEYWORDS

- **Lower Respiratory Tract infections (LRTI):** infection in the lower airways
- **Rapid Diagnosis**: a medical diagnostic test that is quick, easy to perform and provides results in 20 minutes or less.
- **Real-time PCR-based assay:** a method for the detection, quantification and typing of different microbial agents.
- **Respiratory panel:** checks for pathogens (bacteria, virus or other organisms) in the respiratory tract.
- ***Streptococcus pneumoniae:*** gram-positive, lancet-shaped, and facultative anaerobe bacterium
- **Nasopharyngeal swab:** a test used to look for viruses or bacteria that can cause respiratory infections. It takes a sample of cells from the nasopharynx, which is the top part of the throat and nose.

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