Experience with the Quantitative \textit{lytA} Gene Real-time Polymerase Chain Reaction for the Detection of \textit{Streptococcus pneumoniae} from Pediatric Whole Blood in Pakistan

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Abstract

**Background:** We present our experience with optimization and diagnostic use of quantitative real-time polymerase chain reaction (PCR) targeting the \textit{lytA} gene of \textit{Streptococcus pneumoniae} for the detection of \textit{S. pneumoniae} in whole blood of children <5 years of age. The assay was optimized to detect $\geq 5$ CFU/10 $\mu$l or 1 copy of DNA/2 $\mu$l of blood. **Methods:** This assay was applied on 1912 whole blood specimens collected from children <5 years of age with pneumonia, of which 35 specimens were \textit{lytA} positive. The bacterial loads were determined through categorization of load into five different categories, i.e., very high load, high load, moderate load, low load, and very low load. **Results:** Of the 35 \textit{lytA}-positive samples, 9 (25.71%), 4 (11.42%), 1 (2.85%), 13 (37.14%), and 8 (22.85%) were categorized as very high load, high load, moderate load, low load, and very low load, respectively. Extracted samples were also subjected to serotyping by the Centers for Disease Control and Prevention PCR scheme. Positive samples with very high load and high load category were serotyped successfully in all instances. A high proportion of samples with low and very low load (61.53% and 75%, respectively) remained untypeable by the currently proposed schemes. **Conclusions:** \textit{lytA} PCR assay in whole blood provides rapid and sensitive results for the diagnosis of invasive \textit{S. pneumoniae} disease in a resource-limited setting, while also being amenable to quantitation and serotyping.

**Keywords:** Blood, immunochromatography, \textit{lytA} gene, real-time polymerase chain reaction, serotyping, \textit{S. pneumoniae}

Introduction

\textit{Streptococcus pneumoniae} is a major cause of childhood morbidity and mortality globally.\textsuperscript{[1]} Invasive pneumococcal disease (IPD) and pneumococcal pneumonia in children are now considered preventable illnesses following the introduction of pneumococcal conjugate vaccines (PCVs), but reduction in the rates of IPD incidences highly depends on vaccine serotype proportions causing disease.\textsuperscript{[2]}

Laboratory diagnosis of IPD by conventional culture and serological methods has several limitations including but not limited to low sensitivity due to prior antibiotic use and fastidiousness of the organism, longer turnaround time (TAT), and requirement of larger volumes of blood.\textsuperscript{[3]} Hence, improved molecular diagnostic methods (e.g., polymerase chain reaction [PCR]) of targeting the \textit{S. pneumoniae} virulence genes (\textit{psaA}, \textit{ply}, \textit{php}, \textit{lytA}) have been increasingly used.\textsuperscript{[4-8]} More recently, probe-based real-time PCR has been developed with rapid TAT, increased sensitivity, and multiplexing capability.\textsuperscript{[9,10]} \textit{lytA} gene has shown greater utility and increased sensitivity.\textsuperscript{[11,12]} However, the detection of \textit{S. pneumoniae} in blood through real-time PCR has its own limitations such as presence of inhibitors in blood and potential cross-reactivity (\textit{Streptococcus mitis}) is known to harbor low quantities of \textit{lytA} and \textit{ply} genes as reported by several authors.\textsuperscript{[13-17]} Owing to limitations described in literature, this assay has not been widely adopted for routine use in the diagnosis of IPD.

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We optimized a quantitative lytA gene real-time PCR in whole blood and applied it to a large number of whole blood samples from children with pneumonia in a low-resource setting where routine diagnosis through blood cultures is not possible. We also assessed the impact of this assay on serotyping of *S. pneumoniae*. Our secondary aim was to compare this quantitative lytA gene real-time PCR with a rapid immunochromatography testing (ICT-BINAXNOW®) that we conducted on a subset of samples.

**METHODS**

**Generation of standard curve and lytA assay optimizations**

Standard curve was generated by setting up 10-fold serial dilutions of *S. pneumoniae* ATCC 49619 and suspending in healthy donor’s ethylenediaminetetraacetic acid (EDTA) blood to achieve final concentrations of 5 × 10^5–5 × 10^2 CFUs/mL. In brief, serial dilutions of *S. pneumoniae* ATCC 49619 0.5 McFarland (McF) suspension were prepared in 3 ml of phosphate-buffered saline. 190 µl of EDTA blood was spiked with 10 µl of each dilution. To verify assay specificity, blood was also spiked with 0.5 McF suspensions of *S. viridans* with a 5 × 10^6 CFU/mL. DNA was extracted and followed by lytA real-time PCR, as described below [Supplementary Table 1]. Standard curve was generated by plotting the CFU/mL concentrations against the cycle threshold (Ct) values of spiked samples.

**Data sources**

The optimization and routine use were carried out as part of the pneumococcal conjugate vaccine impact study, which is being conducted in five districts of Sindh province in Pakistan. The study was approved by the Aga Khan University Ethical Review Committee (ERC no. 2818-PED-ERC-13). Whole blood samples (*n* = 1912) from children aged <5 years with pneumonia, meeting the WHO criteria, were collected, after obtaining written informed consent from parents or guardians of children. Two milliliters of blood was collected in EDTA tubes. lytA PCR was performed for the identification of *S. pneumoniae* as described below. Blood specimens that were positive for *S. pneumoniae* lytA gene were further serotyped by sequential multiplex conventional or real-time PCR. A subset (*n* = 1271) of blood specimens were also tested with pneumococcal ICT-BINAXNOW® testing, as per manufacturer’s instructions for urine samples.

**DNA extraction from whole blood**

Preliminary blood samples was carried out by treating 200 µl of well-mixed whole blood with 3 µl of 2500U/ml mutanolysin, 20 µl lysozyme (200 mg/ml), 13.5 µl lysostaphin (1.5 mg/ml), and 65 µl TE buffer (pH 8), incubating at 37°C for 30 min, followed by the DNA extraction using QIAamp DNA Blood Mini kit (QIAGEN Inc., Valencia, California, USA) according to manufacturer’s instructions (spin protocol). DNA was archived at −20°C, until further processing.

**Real-time monoplex lytA polymerase chain reaction for the identification of Streptococcus pneumoniae in blood**

Real-time monoplex lytA PCR was carried out by targeting lytA gene, as described by Carvalho Mda et al. in 2007. In brief, a 25 µl PCR reaction mixture was prepared by adding 12.5 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems®, Life Technologies, USA), 1 µl of each forward and reverse lytA primer (10 µM), 0.15 µl of lytA probe (5 µM), 6.35 µl of molecular biology grade nuclease-free water, and finally, 2 µl of template DNA added to the mixture. Thermal cycling was performed, using cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min, reaction was set up in Corbett Rotor-Gene 6000 thermal-cycler (Corbett Life Science, USA). A Ct cutoff ≤35 was set for positive specimens. The assay was optimized to detect 500 CFU/ml of *S. pneumoniae* in 200 µl of whole blood, based on standard curve data.

**RNase P gene monoplex real-time polymerase chain reaction assay used as internal positive control reaction**

**RNase P** gene monoplex real-time PCR was performed independently on every specimen as an internal positive control reaction, to rule out the presence of any inhibitors and false negativity. In brief, 21 primer pairs were grouped into seven triplex reactions for covering 39 primer pairs to cover 68 serogroups/serotypes. PCR was carried out in reaction mixture volumes of 25 µl using Invitrogen-Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen, USA), with 10 µM primers and probes. Amplification was performed on Applied Biosystems® 7500 real-time PCR system (Applied Biosystems®, Life Technologies, USA).

**Pneumococcal serotyping by sequential multiplex conventional/real-time polymerase chain reaction**

*S. pneumoniae* serotyping of those blood samples with lytA Ct value ≥30 was performed by triplex sequential real-time PCR as described by Pimenta et al. In brief, 21 primer pairs were grouped into seven triplex reactions for covering 39 serogroups/serotypes. PCR was carried out in reaction mixture volumes of 25 µl, using Invitrogen-Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen, USA), with 10 µM primers and probes. Amplification was performed on Applied Biosystems® 7500 real-time PCR system (Applied Biosystems®, Life Technologies, USA).

**Immunochromatography-BINAXNOW® testing**

A subset of blood specimens (*n* = 1271) were also subjected to ICT-BINAXNOW® *S. pneumoniae* kit testing (Alere, Hague, the Netherlands) as per manufacturer’s instructions for urine samples.
In brief, a swab was dipped into a whole sample and then inserted into the test device. Three drops of reagent-A solution were added and results were recorded after 15 min. The test was interpreted as positive if both sample and control lines were present, while interpreted as negative if only the control line was present.

**RESULTS**

**Standard curve data**

Standard curve showed a linear relationship between Ct values and CFU/ml, DNA copies/2 µl of the sample [Figure 1]. The limit of detection for the assay was 5 CFU/10 µl, equivalent to 1 copy of DNA/2 µl of the sample. The analytical sensitivity of the assay was found to be 500 CFU/ml of S. pneumoniae in 200 µl of blood. Blood samples spiked with S. viridans were negative for lytA quantitative real-time PCR, showing no cross-reactivity [Supplementary Table 1].

**Result interpretation and quality control**

A clinical whole blood specimen was considered positive for S. pneumoniae when lytA PCR yielded a Ct value ≤35, and the internal control human RNAseP gene was positive at a Ct value of ≤35. PCR-negative control and extraction-negative control yielded no Ct value and remained negative.

**lytA quantitative real-time polymerase chain reaction assay results**

A total of 1912 blood specimens were subjected to lytA quantitative real-time PCR results, of which 35 specimens (1.83%) were found positive. On the basis of standard curve data, five categories with respect to bacterial load were made, i.e., very high load (i.e., >5 × 10^6 CFU/ml), high load (between 5 × 10^5 and 5 × 10^6 CFU/ml), moderate load (between 5 × 10^4 and 5 × 10^5 CFU/ml), low load (between 5 × 10^3 and 5 × 10^4 CFU/ml), and very low load (between 5 × 10^2 and 5 × 10^3 CFU/ml). Of the 35 lytA positives, 9 (25.71%), 4 (11.42%), 1 (2.85%), 13 (37.14%), and 8 (22.85%) were categorized as very high load, high load, moderate load, low load, and very low load, respectively.

**Correlation with serotyping results and Immunochromatography-BINAXNOW®**

Table 1 shows the correlation between lytA real-time PCR quantification with pneumococcal serotypes and ICT-BINAXNOW®.

All specimens with either very high load or high load were successfully serotyped. However, a large proportion of the samples with low load to very low load remained untypeable by the serotypes included in the schemes. One outlier in the moderate load category also remained untypeable.

The overall percentage agreement between ICT-BINAXNOW® and quantitative lytA real-time PCR was found to be 98.74%, while positive percentage agreement and negative percentage agreement were 48.38% and 100%, respectively [Supplementary Table 2]. The lowest detectable limit for a positive ICT-BINAXNOW® was determined to be between 5 × 10^2 and 5 × 10^3 CFU/ml.

**DISCUSSION**

Advancement in nucleic acid detection techniques, particularly probe-based real-time PCR, offers an opportunity to improve the diagnosis of invasive S. pneumoniae disease. Detection of S. pneumoniae is difficult, because it is fastidious, difficult to grow due to autolysis phenomenon, and there is a limited experience and literature available for a real-time PCR diagnosis of IPD in resource-limited settings. We have successfully demonstrated quantitative lytA real-time PCR as well as ICT-BINAXNOW® in a cohort of children <5 years of age with pneumonia. Although lytA real-time PCR does not replace conventional diagnosis, it offers rapid detection of S. pneumoniae in blood and also provides valuable serotyping results which is of paramount importance in the post-PCV-10 era.

Our study revealed that serotyping of S. pneumoniae was dependent on bacterial load, i.e., there was no untypeable S. pneumoniae in very high load (>5 × 10^6 CFU/ml) and high load categories (between 5 × 10^5 CFU/ml and 5 × 10^6 CFU/ml), while the proportion of untypeable results increased in lower load categories. However, the conserved region, i.e., cpsA was present in all the untypeable samples. Therefore, it is likely that these samples were pneumococcal serotypes other than those in our testing repertoire. On the contrary, it is possible that those were truly untypeable, given the 100% success in typing those with low Ct value for lytA, it is more likely that

![Figure 1: Line graph showing the comparison of cycle threshold values in quantitative lytA real-time polymerase chain reaction on Y-axis and CFU/ml (in the upper image) or DNA copies/2 µl (in the lower image) on X-axis. Trend line showing a linear relationship between the cycle threshold values and CFU/ml or DNA copies/2 µl TAC. Depicting a linear relationship of cycle threshold values versus CFU/ml, DNA copies/2 µl on a standard curve. CFU/ml: colonies forming unit/milliliter, DNA copies/µl: deoxyribonucleic acid/microliter](Image)
Table 1: Relationship of quantitative lytA real-time polymerase chain reaction with immunochromatography-BINAXNOW® and pneumococcal serotyping

<table>
<thead>
<tr>
<th>Category</th>
<th>Pneumococcal load (CFU/ml)</th>
<th>Pneumococcal load (DNA copies/2ul)</th>
<th>Ct value Range</th>
<th>lytA positive (n)</th>
<th>ICT-BINAX Agreement with lytA positive</th>
<th>Proportion of Serotypes Typed Successfully n (%)</th>
<th>Proportion of Serotypes Not Typed (Untypeable) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very High load</td>
<td>&gt;5×10^6 CFU/ml</td>
<td>&gt;1×10^6 DNA copies/2ul</td>
<td>≤23.87</td>
<td>9</td>
<td>Positive=07 Negative=02 Positive percent agreement=100.00%</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>High load</td>
<td>Between 5×10^5 and 5×10^6 CFU/ml</td>
<td>Between 1×10^4-1×10^5 DNA copies/2ul</td>
<td>23.88-27.88</td>
<td>4</td>
<td>Positive=03 Negative=01 Positive percent agreement=77.77%</td>
<td>4 (100.00%)</td>
<td>Nil</td>
</tr>
<tr>
<td>Moderate load</td>
<td>Between 5×10^4 and 5×10^5 CFU/ml</td>
<td>Between 1×10^3-1×10^4 DNA copies/2ul</td>
<td>27.89-31.08</td>
<td>1</td>
<td>Positive=00 Negative=01 Positive percent agreement=0%</td>
<td>Nil</td>
<td>1 (100.00%)</td>
</tr>
<tr>
<td>Low load</td>
<td>Between 5×10^3 and 5×10^4 CFU/ml</td>
<td>Between 1×10^2-1×10^3 DNA copies/2ul</td>
<td>31.09-33.67</td>
<td>13</td>
<td>Positive=04 Negative=06 Not Performed=03 Positive percent agreement=38.46%</td>
<td>5 (38.46%)</td>
<td>8 (61.53%)</td>
</tr>
<tr>
<td>Very Low load</td>
<td>Between 5×10^2 and 5×10^3 CFU/ml</td>
<td>Between 1×10^1-1×10^2 DNA copies/2ul</td>
<td>33.68-36.85</td>
<td>2</td>
<td>Positive=01 Negative=06 Not Performed=01 Positive percent agreement=25.00%</td>
<td>2 (25.00%)</td>
<td>6 (75.00%)</td>
</tr>
</tbody>
</table>

A total of 1912 blood specimens were performed for lytA real-time PCR, of which 35 were found positive for S. pneumoniae and a total of 1271 blood specimens were processed for ICT-BINAXNOW®, of which 15 were found positive for S. pneumoniae. PCR: Polymerase chain reaction, S. pneumoniae: Streptococcus pneumoniae, ICT: Immunochromatography

we need more DNA copies for serotyping to work. We intend to resolve these untypeable results through whole-genome sequencing in the future.

Our study has limitations, since this was a field study, blood cultures could not be performed and thus quantitative lytA real-time PCR results could not be compared with a reference test. Furthermore, lytA PCR was not performed in blood samples from healthy children, and therefore we could not determine its diagnostic specificity.

CONCLUSION
There is a dire need to evaluate sensitive and rapid assays for a resource-limited setting that can detect S. pneumoniae in blood and will provide a strong foundation for pneumococcal serotyping, and we have described such assay. lytA PCR assay facilitated the diagnosis of IPD in our cohort. Once diagnostic specificity is demonstrated in a healthy cohort of children, this assay can be successfully used for the diagnosis of IPD in children and can be adapted to point-of-care platforms to facilitate rapid field diagnosis.

Acknowledgments
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Conflicts of interest
There are no conflicts of interest.

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5. Gillespie SH, Ullman C, Smith MD, Emery V. Detection of Streptococcus
Kabir, et al.: Assessment of quantitative lytA real-time PCR for *S. pneumoniae*


### Supplementary Table 1: Optimization of quantitative *lytA* real-time polymerase chain reaction, with reference to standard curve

<table>
<thead>
<tr>
<th>S#</th>
<th>Dilution Preparation</th>
<th>Dilution</th>
<th>CFU/ml</th>
<th>CFU/10ul on SBA</th>
<th>CFU/ml</th>
<th>Copies Of Pneumococcal DNA/2ul</th>
<th>Ct Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5 McFarland suspension of <em>S.pneumoniae</em> in 3ml PBS</td>
<td>Neat</td>
<td>5×10⁶ CFU/ml</td>
<td>50000 CFU/10ul</td>
<td>5,000,000 CFU/ml</td>
<td>10,000</td>
<td>23.88</td>
</tr>
<tr>
<td>2</td>
<td>1 ml from neat +9ml PBS</td>
<td>1:10</td>
<td>5×10⁵ CFU/ml</td>
<td>5000 CFU/10ul</td>
<td>500000 CFU/ml</td>
<td>1000</td>
<td>27.89</td>
</tr>
<tr>
<td>3</td>
<td>1ml from 1:10+9ml PBS</td>
<td>1:100</td>
<td>5×10⁴ CFU/ml</td>
<td>500 CFU/10ul</td>
<td>50000 CFU/ml</td>
<td>100</td>
<td>31.09</td>
</tr>
<tr>
<td>4</td>
<td>1ml from 1:100+9ml PBS</td>
<td>1:1000</td>
<td>5×10³ CFU/ml</td>
<td>50 CFU/10ul</td>
<td>5000 CFU/ml</td>
<td>10</td>
<td>33.68</td>
</tr>
<tr>
<td>5</td>
<td>1ml from 1:1000+9ml PBS</td>
<td>1:10000</td>
<td>5×10² CFU/ml</td>
<td>5 CFU/10ul</td>
<td>500 CFU/ml</td>
<td>1</td>
<td>36.85</td>
</tr>
<tr>
<td>6</td>
<td>1ml from 1:10000+9ml PBS</td>
<td>1:100000</td>
<td>5×10¹ CFU/ml</td>
<td>0</td>
<td>No Growth</td>
<td>0</td>
<td>Neg</td>
</tr>
<tr>
<td>7</td>
<td>0.5 McFarland suspension of <em>S.viridans</em> in 3ml PBS</td>
<td>Neat</td>
<td>5×10⁶ CFU/ml</td>
<td>50000 CFU/10ul</td>
<td>5,000,000 CFU/ml</td>
<td>0</td>
<td>Neg</td>
</tr>
</tbody>
</table>

### Supplementary Table 2: Estimation of agreement between immunochromatography-BINAXNOW® and quantitative *lytA* real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Estimation of Agreement</th>
<th>Quantitative lytA real-time PCR (Non-reference Standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>ICT-BINAX (New test)</td>
<td>a (15)</td>
</tr>
<tr>
<td>Positive</td>
<td>c (16)</td>
</tr>
<tr>
<td>Negative</td>
<td>a + c (31)</td>
</tr>
</tbody>
</table>

*overall percent agreement=98.74% {reference formula used: 100% x (a + d)/(a + b + c + d)}, * positive percent agreement=48.38% {reference formula used: 100% x a/(a + c)}, * negative percent agreement=100.00% {reference formula used: 100% x d/(b + d)}