Original article

Impact of Laboratory Methods and Gene Targets on Detection of *Streptococcus pneumoniae* in Isolates and Clinical Specimens

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Abstract

Background: Timely identification of *Streptococcus pneumoniae* infections can lead to a decrease in mortality rates. Differentiation of *S. pneumoniae* from other similar species using traditional culture-based and molecular methods is problematic. In this study, we assessed the efficacy of identifying the *blpA* and *lytA* for the detection of *S. pneumoniae* from isolates and various clinical samples using molecular methods.

Methods: A total of 440 clinical samples were collected from patients with suspected invasive pneumococcal infections during February 2016 to October 2018. Biochemical tests were used to confirm the dubious colonies on 5% sheep blood agar. Fifty-seven confirmed isolates, 57 culture-positive samples, and 57 culture-negative samples were analyzed for the presence of *blpA* and *lytA* using both conventional and real-time PCR.

Results: All the isolates and culture-positive samples were positive for *blpA* and *lytA* by both PCR methods. Of the 57 culture-negative samples, conventional and real-time PCR amplified *blpA* from six and two samples, and *lytA* from seven and two samples, respectively.

Conclusions: The specificity of real-time PCR assay was significantly higher than that of conventional PCR for the identification of *S. pneumoniae*. In addition, it is suggested that respiratory secretions are not suitable specimen for direct diagnosis of pneumococcal infections.

Keywords: *blpA*, Molecular Detection, Molecular Diagnostic Techniques, Real-Time Polymerase Chain Reaction, *Streptococcus pneumoniae*.

Introduction

Invasive pneumococcal diseases (IPD), which include bacterial pneumonia, meningitis, and sepsis in children and seniors (1), are caused by *S. pneumoniae* and are major infectious diseases. A recent survey estimated that *S. pneumoniae* was responsible for 393,000 pneumonia-related deaths in children under age five in 2015 (2). Nasopharyngeal colonization by *S. pneumoniae* frequently leads to IPD and this organism is a reservoir of genes for transmission to other related species (3). Hence, correct diagnosis of pneumococcal infections is critical to understanding the potential for disease in a community-based population and providing timely treatment.

Pneumococcal isolates are heterogeneous and have the ability for genetic alteration and adaptation. These abilities result in the protection of bacteria in multiple conditions. Antibiotic-resistant strains have emerged due to genetic

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mutations and are spreading. Moreover, the current vaccine, which protects against some serotypes, may not be effective against emerging vaccine-escape strains (4).

Culture-based methods are the gold standard for the identification of *S. pneumoniae* from clinical specimens (5). However, these methods have some deficiencies, including diminished sensitivity due to antibiotic therapy, activation of autolysins during sample transportation, and the emergence of optochin-resistant strains, which can give false-negative results (6, 7). Therefore, the evaluation of new methods for rapid and reliable identification is an important research area. In recent years, several genes, including *ply* and *lytA*, have been targeted for the detection of pneumococci by molecular methods. However, it has been shown that the use of these genes can give false-positive and false-negative results (8). Consequently, the evaluation of new conserved genes is crucial for the detection of *S. pneumoniae* in clinical specimens. In the present study, we assessed the sensitivity and specificity of the *blpA* as a conserved gene for the detection of *S. pneumoniae* from isolates and clinical samples and compared it with the *lytA* gene (*lytA)*, as recommended by the World Health Organization (WHO) (9).

Materials and methods
Clinical isolates
From February 2016 through October 2018, 440 clinical specimens were collected from patients with clinically suspected invasive pneumococcal infections. Clinical specimens were obtained from blood, cerebrospinal fluid (CSF), tracheal aspirates, ascites, pleural aspirates, bronchoalveolar lavage (BAL), sputum, sinus secretions, and eye infections. These samples were inoculated onto 5% sheep blood agar plates and incubated at 37 °C in 5% CO_2 for 24 hr. The *S. pneumoniae* colonies were identified using standard conventional tests including gram staining, catalase, bile solubility, and optochin (MAST, UK) sensitivity tests. The isolates were preserved in trypticase soy broth (Merck, Germany) with 10% glycerol at -70 °C for further studies (10).

DNA extraction
Genomic DNA was extracted from isolates and clinical samples using a DNeasy Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

PCR assay
All of the isolates, 57 culture-positive, and 57 culture-negative samples were analyzed for the presence of *blpA* and *lytA* with the primers shown in Table 1. Culture-negative samples were randomly chosen from 383 specimens, including sputum (n= 17), blood (n= 13), BAL (n= 9), tracheal aspirates (n= 7), CSF (n= 6), synovial fluid (n= 3), and ascitic fluid (n= 2). PCRs were performed separately for *lytA* and *blpA* with hot start Taq master mix kit (Sinaclon, Iran) that contained 12.5 µl of 2x hot start Taq master mix (including 0.4 mM of each dNTP, 3mM $MgCl_2$, 0.08 U/µl Taq DNA polymerase), 1µl of each primer (10 pmol/µl), 2 µl of DNA, and 11.5 µl of ddH_2O. The PCR program included an initial denaturation at 94 °C for 5 min, 35 amplification cycles of 94 °C for 30 sec, 58 °C for 35 sec, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were electrophoresed in 1% agarose gels (Biotium Inc. USA).

Table 1. PCR primer sequences.

Primers	Sequences	Product sizes (bps)	Reference
blpA	F: 5′-GCTACTAGCGGTCTTGATGTC-3′ R: 5′-AGAACAATGACACGGTTGGTT-3′	131	This Study
lytA	F:5′-ACGCAATCTAGCAGATGAAGCA-3′ R:5′-TCGTGCGTTTTAATTCCAGCT-3′	319	(11)

Real-time PCR assay
The real-time PCR assays targeting *blpA* and *lytA* were performed separately with Taq Man probe and HiFi hot start master mix in a final volume of 20 µl containing 4 µl of master mix, 1 µl of each primer (10 pmol/µl) (Table 2), 1 µl of probe (10 pmol/µl), 2 µl of DNA and 11 µl of ddH_2O. The *S. pneumoniae* strain ATCC 49619 was included as a control. This method was used for the isolates, culture-positive (n= 57), and our selected culture-negative samples (n= 57). The

amplification program is shown in Table 3. To determine the sensitivity and limit of detection (LOD), we initially prepared serial 10-fold dilutions of 0.5 McFarland standard of *S. pneumoniae*. The dilution series was inoculated on sheep blood agar plates and colonies were counted (in CFU). A dilution series of purified DNA (10^7 CFU) was prepared and the concentration of primers and probes optimized following DNA amplification. The curve of threshold cycle (Ct) values was plotted based on the number of bacteria.

Table 2. Real-time PCR primer and probe sequences.

Primers and probes		Sequences	References
blpA primers	F: 5′-GCTACTAGCGGTCTTGATGTC-3′ R: 5′-AGAACAATGACACGGTTGGTT-3′		This Study
blpA probe	5′-FAM-AGCCCATCGTCTCAGTATAGCCGAACG-3′-BHQ1		This Study
lytA primers	F:5′-ACGCAATCTAGCAGATGAAGCA-3′ R:5′-TCGTGCGTTTTAATTCCAGCT-3′		(12)
lytA probe	5′-FAM-TGCCGAAAACGCTTGATACAGGGAG-3-′BHQ1		(12)

Table 3. Temperature program for *blpA* and *lytA* real-time PCR.

Step	Temperature	Time	Cycle No.
Initial Denaturing	95 °C	10 Min	1
Denaturing	95 °C	15 Sec	40
Annealing and Extension	60 °C	60 Sec	40

Results

Bacterial isolates

Fifty-seven strains of pneumococci were isolated from 440 clinical samples using culture-based methods, including gram staining, bile solubility, and optochin susceptibility tests. Two isolates were optochin-resistant, and identified as *S. pneumoniae* strains by molecular methods.
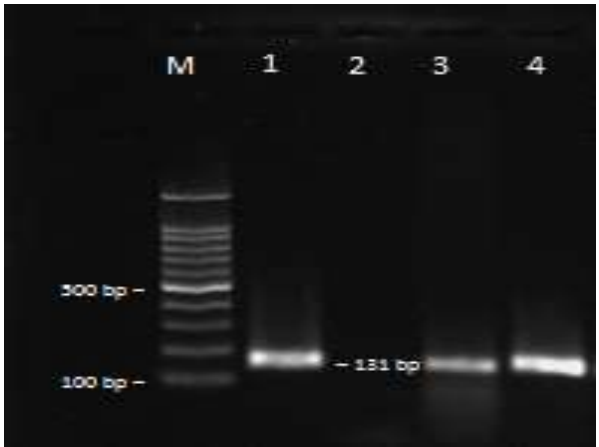
Conventional PCR assay

By conventional PCR, all the isolates and culture-positive specimens were positive for *lytA* and *blpA*, while six (10.5%) and seven (12.3%) of the culture-negative samples were positive for *blpA* and *lytA*, respectively (Figs. 1 and 2). Moreover, two of these samples were positive for both. All the PCR-positive, culture-negative samples were from respiratory secretions.
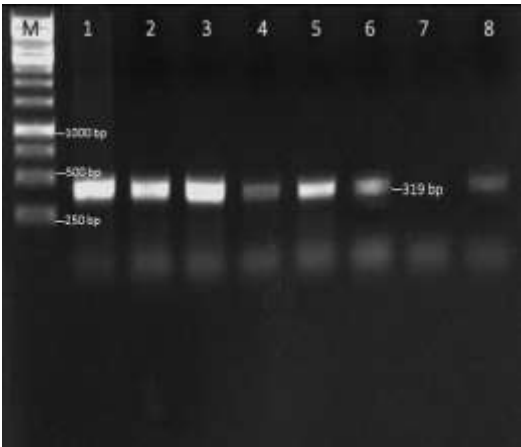

Fig. 1. Amplification of *blpA* from *S. pneumoniae*. M: DNA marker (100 bps); 1: positive control; 2: negative control; 3 and 4: samples.

Fig. 2. Amplification of *lytA* from *S. pneumoniae*. M: DNA marker (1 Kbps); 1: positive control; 2, 3, 4, 5, 6 and 8: samples; 7: negative control.

Real-time assay

Using real-time PCR, we identified both genes with low Ct values in all the isolates and culture-positive samples, in agreement with the conventional PCR results. A standard curve was obtained from *S. pneumoniae* ATCC 49619 (Fig. 3). The average Ct values of the *blpA* and *lytA* real-time PCR for culture-positive samples were 25.8 (18.8 to 34.2) and 24.6 (18.4 to 33.8), respectively (Fig. 4A). Two of the 57 (3.5%) culture-negative samples were positive for *blpA,* two (3.5%) were positive for *lytA*, and one of those samples was positive for both genes. Ct values were 32.8 and 34.1 for these two *blpA* culture-negative samples, (Fig. 4B), and 31.6 and 33.4 for the two *lytA* samples. The LODs for both *blpA* and *lytA* were determined to be 10 *S. pneumoniae* cells per milliliter.
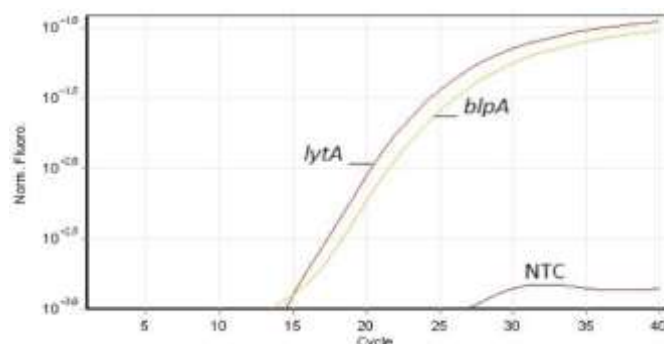

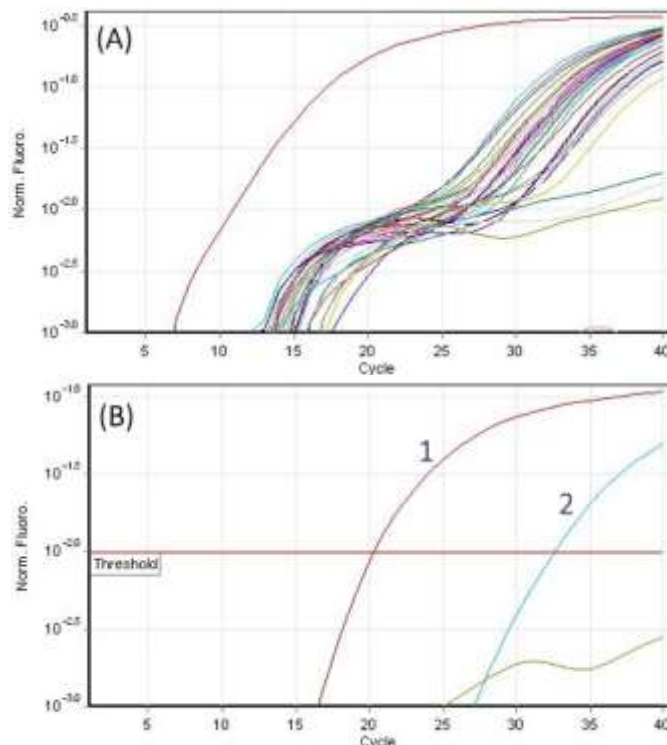**Fig. 3.** Real-time PCR results of *lytA* and *blpA* from *S. pneumoniae* strain ATCC 49619.

Fig. 4. Real-time PCR results for *blpA*; **A:** clinical samples, **B:** number 1 is *blpA* from positive control, number 2 is *blpA* from one culture-negative sample, which gave a positive result in the PCR.

Discussion

In our study, two isolates were resistant to optochin disk. This test is broadly used to differentiate pneumococcal isolates from other streptococci (13). Optochin-resistant *S. pneumoniae* isolates have been reported in previous investigations (14, 15, 16). In most cases, resistance is due to point mutations in *atpC*, which encodes an ATPase protein subunit (14, 17). The differentiation of *S. pneumoniae* from other related species is essential to choose the appropriate therapeutic strategies, due to the increasing resistance to penicillin and macrolides among pneumococcal and other closely-related species (6, 18). We demonstrated that the emergence of optochin-resistant isolates could disrupt the efficiency of conventional tests in diagnosing *S. pneumoniae* infections. The administration of antibiotics before sample collection, and activation of autolysins during sample transfer, can lead to false-negative results (8, 19, 20). Recently, several molecular methods for diagnosis of pneumococcus in biological samples have been introduced by targeting *sodA*, *rpoA*, *spn9802*, *psaA*, 16S rDNA, *recA*, *piaA*, *tuf*, *ply,* and *lytA* (6, 21). Although these molecular targets are useful for the detection of pneumococci, they are not able to differentiate *S. pneumoniae* from other closely-related species (12). In previous studies, false-positive results of *lytA* and false-negative results of *ply* and *spn9802* have been shown (20, 22). Real-time PCR targeting *lytA* is currently recommended by the WHO for the detection of pneumococcal DNA in clinical specimens (22). Another diagnostic difficulty is related to the number of microorganisms present in clinical samples that may lead to false-negative results (23). In the present study, 57 culture-positive samples carrying *blpA* and *lytA* were detected using conventional and real-time PCR with low Ct values. There is a concordance between these two molecular methods and it has been demonstrated that both methods are sensitive for detection of pneumococcal DNA in culture-positive samples (24, 25). Our results revealed that the main

difference between these two molecular methods was the ability to detect pneumococcal DNA in culture-negative samples. Using the real-time PCR, two of the 57 culture-negative samples were positive for both *lytA* and *blpA*, while by conventional PCR, six culture-negative samples were positive for *blpA*, seven for *lytA*, and two of these were positive for both genes, indicating false-positive results. Considering the culture method as the gold standard for pneumococci detection, the sensitivity and specificity of conventional PCR for *blpA* were 100 and 89.47% and for *lytA*, 100 and 87.71%, respectively, while the sensitivity and specificity of real-time PCR for both genes were 100 and 96.49%, respectively. The culture-negative samples that gave PCR-positive results were respiratory specimens, indicating that such specimens are not suitable for PCR-based pneumococcal detection methods. Previous studies have also shown that molecular methods are cannot distinguish between colonization and pneumococcal pneumonia (26). The molecular assays are also currently unable to substitute for traditional culture methods due to limitations in the simultaneous detection of multiple pathogens and antibiotic susceptibility (27). Nonetheless, real-time PCR is valuable for the direct detection of pneumococcus from clinical samples and molecular epidemiology studies (28).

Our results indicated that both molecular methods had high diagnostic sensitivity and can be used for the identification of *S. pneumoniae* directly on clinical samples from culture-negative patients but real-time PCR appears more specific than conventional PCR assay. In addition, respiratory secretions were found to be unreliable specimens for the identification of *S. pneumoniae* as they gave false-positive results by both methods.

Acknowledgment

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