Jundishapur J Microbiol. 2017 June; 10(6):e14378. doi: 10.5812/jjm.14378.
Published online 2017 June 17. **Research Article**

Evaluation of *lytB* Gene for Detection of *Streptococcus pneumoniae* in Isolates and Clinical Specimens by Real-Time PCR

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Received 2016 November 13; **Revised** 2017 April 13; **Accepted** 2017 May 06.

Abstract

Background: *Streptococcus pneumoniae* is a causative agent of morbidity and mortality worldwide. Diagnosis of pneumococcal infection includes conventional culture-based and molecular methods. Differentiation of *S. pneumoniae* from other mitis group streptococci is not reliable.
Objectives: We aimed to evaluate the efficacy of *lytB* gene along with *lytA* gene for detection of *S. pneumoniae* in isolates and clinical samples using conventional and real-time PCR methods.
Methods: In this cross-sectional study, a total of 560 clinical specimens were collected from patients during February-September 2015. The samples were cultured on 5% sheep blood agar and suspected colonies were identified by biochemical assays. The antibiotic susceptibility test was performed by disk diffusion and serial microdilution methods. 46 culture-negative and 46 culture-positive samples were examined to evaluate the presence of *lytA* and *lytB* genes using conventional and real-time PCR methods.
Results: A total of 46 (8.2%) isolates of *S. pneumoniae* were identified in suspected specimens. 52% (24/46) of isolates exhibited multiple drug resistance (MDR). All 46 isolates contained both *lytA* and *lytB* genes. Real-time PCR assay detected both genes with low CT values in culture-positive samples. Among culture negative specimens, one sample was positive for both the genes.
Conclusions: The *lytB* is similar to *lytA* in sensitivity for diagnosis of *S. pneumoniae* in isolates and clinical samples based on both the molecular methods. The results confirmed the applicability of real time PCR based on *lytB* genes for detection of *S. pneumoniae*.

Keywords: Real-Time PCR, Antibiotic Resistance, *Streptococcus pneumonia*, *lytA*, *lytB*

1. Background

Streptococcus pneumoniae is a major causative agent of morbidity and mortality worldwide (1). The world health organization (WHO) estimates that annually 1.6 million people die of pneumococcal disease (2). The introduction of conjugated vaccines has declined the number of deaths due to pneumococcal infections, especially among children. However, pneumococcal infections remain an important disease among children under 2 years-old, immune compromised people, and the elderly aged above 65 years (3).

Accurate identification of *S. pneumoniae*can track the organ-based infection. A robust method for detection of *S. pneumoniae* can decrease the morbidity and mortality rate of pneumococcal infections, especially in developing countries. Therefore, a reliable method is essential to accurately treat patients and control pneumococcal disease (3, 4).

Diagnosis of pneumococcal infection includes conventional culture-based and molecular methods. The culture-based methods include optochin susceptibility, agglutination, and bile solubility, which are routinely used for differentiation of *S. pneumoniae* from other species such as *S. viridans*. However, these assays can lead to misidentification of this agent due to the emergence of optochin resistant pneumococcus (5). Furthermore, antibiotic therapy before sample collection or activation of autolysins during sample transmission can lead to false negative results (5, 6).

Latex agglutination, counter immunoelectrophoresis, and immunochromatography have low sensitivity for detection of *S. pneumoniae* (7). Recently, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) proposed for rapid diagnosis of mitis group streptococci sometimes has produced inaccurate results (8). Therefore, molecular methods have been developed to accurately diagnose *S. pneumoniae* in isolates and clinical samples.

Molecular methods have higher sensitivity for the detection of *S. pneumoniae* among patients under antibiotics

therapy (9). They are used for diagnosis of pneumococcal infections based on identical genes i.e.: pneumolysin gene (*ply*), autolysin gene (*lytA*), and *spn9802*. Differentiation of *S. pneumoniae* is not reliable by detection of these genes in the genus (8). Thus, it is essential to use species-specific genes for accurate diagnosis of *S. pneumoniae* from clinical samples.

2. Objectives

In the present study, we aimed to evaluate the efficacy of *lytB* gene along with *lytA* gene for detection of *S. pneumoniae* in isolates and clinical samples using conventional and real-time PCR methods.

3. Methods

3.1. Clinical Isolates

In this cross-sectional study, a total of 560 clinical specimens were collected from patients with suspected pneumococcal infections in teaching hospitals of Tehran during February-September 2015. The specimens were cerebrospinal fluid, ascites fluid, trachea aspirates, blood, pleural fluid, sputum, bronchoalveolar lavage, ear discharge, synovial fluid, and eye discharge. The mentioned samples were cultured on 5% sheep blood agar (Merck, Germany) and incubated in 5% CO_2 at 37°C for 24 hours. Confirmation of suspected *S. pneumoniae* colonies was performed by gram staining, bile solubility, and optochin (MAST, UK) susceptibility tests. All isolates were cultured in trypticase soy broth (Merck, Germany) containing 10% glycerol (Merck, Germany) and stored in -70°C (10). This study was approved by the ethics committee of Tehran University of Medical Sciences (code: 26772).

3.2. Antibiotic Susceptibility Testing

The antibiotic susceptibility test was performed using Kirby-Bauer disk diffusion method on Mueller Hinton agar (Merck, Germany) containing 5% sheep blood for the following antibiotics: vancomycin (30 μg), tetracycline (30 μg), clindamycin (2 μg), cotrimoxazole (25 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), and erythromycin (15 μg) (MAST, UK). Following the 24-hour incubation at 37°C, the inhibition zones were measured according to the guidelines of clinical and laboratory standards institute (CLSI) and reported as resistant (R), intermediate (I), and susceptible (S) (11). Antibiotic susceptibility test for penicillin and cefotaxime was performed using minimum inhibitory concentration (MIC) determination by serial microdilutions method according to CLSI guidelines (11). *Streptococcus pneumoniae* ATCC49619 were used for quality control. All statistical analyses were performed using SPSS, version 22.

3.3. DNA Extraction

DNA of the isolates and clinical samples was extracted by DNA extraction kit according to the manufacturer's instructions (DNeasy Blood and Tissue Qiagen, Germany). The extracted DNA was stored at -20°C.

3.4. PCR Assay

46 isolates were examined to evaluate the presence of *lytA* and *lytB* genes with primers depicted in Table 1 (12). Final volume of reaction was 25 μL hot start Taq master mix kit (Sinaclon, Iran) that included: 12.5 μL of 2x hot start Taq master mix (containing 3 mM $MgCl_2$, 0.4 mM of each dNTP, 0.08 U/μL Taq DNA polymerase), 2 μL DNA, 1 uL of each primer (10 pmol), and 11.5 μL ddH_2O. Initial step was performed at 94°C (5 minutes), 35 amplification cycles of 94°C (30 seconds), 58°C for both the genes (35 sec), 72°C (1 minute), and final extension at 72°C (10 minutes). The PCR products were electrophoresed on 1% agarose gel (Biotium Inc, USA).

3.5. Real-Time PCR Assay

The real-time PCR assays were performed using TaqMan Universal PCR master mix on isolates, 46 culture-negative specimens (46/514) and 46 culture-positive specimens. Culture-negative samples were randomly selected from 514 specimens. The real-time *lytB* and *lytA* PCR assays were performed in a final volume of 20 μL by hot start HiFi real-time PCR master mix consisting of 4 μL hot start master mix, 1 μL each primer (10 pmol) (Table 2) (13), 1 μL probe (10 pmol), 2 μL DNA and 11 μL ddH_2O. *Streptococcus pneumoniae* ATCC49619 was used as the positive control. The applied amplification program is listed in Table 3.

4. Results

4.1. Bacterial Isolates

A total of 46 isolates of *S. pneumoniae* were isolated from 560 clinical specimens by biochemical assays. All the 46 isolates were positive in bile solubility test and all of them except one isolate were susceptible to optochin. The optochin resistant isolates were confirmed as *S. pneumoniae* by both conventional and real time PCR methods.

Table 1. Sequences of PCR Primers

Primers	Sequences	Products Size, bp	Ref.
lytA	F:5′-ACGCAATCTAGCAGATGAAGCA-3′	319	(12)
	R:5′-TCGTGCGTTTTAATTCCAGCT-3′		
lytB	F:5′-ACAGAGGAAGAAGGTTGATGAAG-3′	150	This study
	R: 5′-ACTCATTCGCTGCTTGACTG -3′		

Table 2. Sequences of Real-Time PCR Primers and Probes

Primers and Probes	Sequences	Ref.
***lytA* primers**	F:5′-ACGCAATCTAGCAGATGAAGCA-3′	(13)
	R:5′-TCGTGCGTTTTAATTCCAGCT-3′	
***lytA* probe**	5′-FAM-TGCCGAAAACGCTTGATACAGGGAG-3-′BHQ1	(13)
***lytB* primers**	F:5′-ACAGAGGAAGAAGGTTGATGAAG-3′	This study
	R: 5′-ACTCATTCGCTGCTTGACTG -3′	
***lytB* probe**	5′-FAM-AGCTAGTCCAGAGGGTGCAATGGCT-3′-BHQ1	This study

Table 3. Temperature Program for *lytA* and *lytB* Real-Time PCR Assays

Step	Temperature, °C	Time	Cycle. No, X
Initial Denaturing	95	10 Minutes	1
Denaturing	95	Seconds 15	40
Annealing + Extension	60	Seconds 60	40

Table 4. Pattern of Antibiotic Resistance in 46 *S. pneumoniae* Isolates

Antibiotics	Number of Resistant Isolates (%)
Vancomycin	0
Ciprofloxacin	6 (13.04)
Penicillin (non-meningitis)	7 (16.7)
Chloramphenicol	8 (17.39)
Cefotaxime (non-meningitis)	10 (23.8)
Clindamycin	19 (41.3)
Tetracycline	24 (52.17)
Erythromycin	25 (54.34)
Cotrimoxazole	36 (78.26)

4.2. Antibiotic Susceptibility Test

52% (24/46) of the isolates were non-susceptible to at least 3 antibiotics and exhibited multiple drug resistance (MDR) (14). The isolates with resistance to cotrimoxazole, erythromycin, and tetracycline were the most cases of MDR. Antibiotic resistance profile of the isolates is shown in Table 4. Isolates causing non-meningitis infections were resistant to penicillin (MIC $\geq$ 8 μg/mL). Moreover, 3 of the non-meningitis isolates were intermediate resistant pneumococci (MIC = 4 μg/mL). Overall, 10 (23.8%) non-meningitis isolates were penicillin-non-susceptible pneumococci (PNSP). Moreover, 2 out of 4 meningitis isolates with penicillin MICs of 0.12 μg/mL and 0.5 μg/mL were penicillin-resistant according to CLSI guidelines (11). 10 non-meningitis isolates were cefotaxime-resistant (MIC $\geq$ 4 μg/mL), 2 were intermediate resistant (MIC = 2 μg/mL), and 2 meningitis isolates were cefotaxime-resistant (MI $\geq$ 2 μg/mL).

4.3. Molecular Assays for Detection of S. pneumoniae

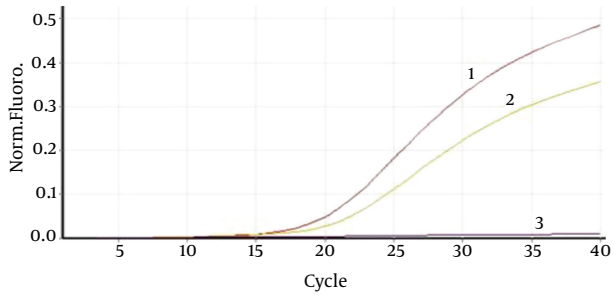
All the isolates, which had been identified biochemically, were confirmed by the conventional and real-time PCR of *lytA* and *lytB* genes. Moreover, one isolate was optochin-negative, but molecular assay of both genes confirmed it as *S. pneumoniae*. Totally, 46 isolates were identified as pneumococci by real-time *lytB* and *lytA* PCR assays. PCR assays were performed on the isolates and the obtained results were concordant with those of real-time PCR.

Since the real-time *lytB* and *lytA* PCR assays performed well on the isolates, we evaluated the diagnostic performance of the primers for the detection of *S. pneumoniae* in clinical specimens. The real-time PCR was done on 46 culture-positive and 46 culture-negative specimens. All the 46 culture-positive samples were positive in both real-time PCR assays with low CT values. Therefore, this is indicative

of the presence of *S. pneumoniae* in the specimens. The median CT values of the *lytB* and *lytA* PCR assays were 23.6 (CT range 18.6 to 30.2) and 22.8 (CT range 18.1 to 29.8), respectively.

Forty six culture-negative samples were randomly selected for real-time PCR assays including: trachea (n = 4), blood (n = 12), plural fluid (n = 6), sputum (n = 15), and pulmonary lavage (n = 9). Among 46 culture-negative specimens, one sample (sputum) was positive for both the genes. The sample belonged to a patient who received antibiotic. CT values of the real-time *lytB* and *lytA* assays for this sample were 33.6 and 32.8, respectively (Figure 1).

Figure 1. Real-Time PCR Results



Streptococcus pneumoniae (ATCC49619), number 1 is *lytA* gene, number 2 is *lytB* gene, and number 3 is negative control.

5. Discussion

In this study, all biochemically identified *S. pneumoniae* isolates were confirmed by conventional and real-time PCR assays of *lytA* and *lytB* genes. One of the isolates was resistant to optochin, confirmed as *S. pneumoniae* by molecular assays. Use of conventional methods for diagnosis of *S. pneumoniae* has limitations due to emergence of optochin resistant pneumococci, time-consuming, and false negative results (6). In previous studies, it has been shown that sensitivity of *lytA* real-time PCR assay in the diagnosis of *S. pneumoniae* isolates is valid (7, 8, 13). In our study, all the isolates identified in biochemical assays were also confirmed by PCR and real-time PCR assays. Thus, the sensitivity of the real-time *lytB* assay was similar to that of real-time *lytA* assay for the diagnosis of *S. pneumoniae* isolates.

Close species of pneumococci such as *S. pseudopneumoniae*, *S. mitis*, and *S. oralis* can colonize in nasopharynx. Transmission of genetic elements between pneumococci and close species can occur, leading to misidentification of these species (15). Commonly used genes for the diagnosis of *S. pneumoniae* such as *lytA*, ply and *spn9802* exist in other members of the viridans group streptococci, affecting the specificity of the molecular assays (16). Furthermore, low levels of bacteria can cause false-negative results in direct detection of clinical samples. This problem can be solved using sensitive molecular assays such as real-time PCR and improved methods of DNA purification (17).

During recent years, the presence of lytA gene has been proposed for diagnosis of *S. pneumoniae*. The strategy of real-time PCR targeting the *lytA* gene has been advised by WHO for detection of pneumococcal DNA in clinical samples (13, 15). However, *lytA* gene has homology among pneumococci and non-pneumococci isolates, which can lead to misidentification of *S. pneumoniae*. The homology is due to point mutations and deletion mutations in the *lytA* gene (15). Previously, two copies of *lytA* gene were characterized in the genome of *S. pseudopneumoniae* isolated from clinical samples (17).

Altogether, the specificity of *lytA* real-time PCR for the diagnosis of *S. pneumoniae* is doubtful and despite the common target gene, there have been various results. Several studies have shown that this method is specific, but some others have not confirmed these findings (13, 18). It has been shown that this method cannot differentiate *S. pneumoniae* from *S. pseudopneumoniae* isolates. For the differentiation of these two species, the *spn9802* gene has been proposed. Although this gene can differentiate *S. pneumoniae* from *S. pseudopneumoniae*, in direct examination from clinical samples it is not applicable (8).

Currently, *lytA* real-time PCR assay is an interesting strategy for direct detection of *S. pneumoniae* in clinical samples that needs the association with a second gene (19). In the present study, the real-time PCR assay was done on 46 culture-negative and 46 culture-positive specimens. All the culture-positive samples (46/46) were positive for these genes and one of the culture-negative samples (1/46) was positive for both genes. None of these two genes were observed in the remaining 45 samples. Overall, the both genes were similar for detection of *S. pneumoniae* in isolates and clinical samples. More studies need to perform on streptococcal strains by *lytB* gene in order to more fully evaluate the specificity of this gene.

In this study, 23.8% of non-meningitis isolates were penicillin non-susceptible, which is similar to studies from other parts of the world. Penicillin non-susceptible isolates in Turkey, North America, and Europe were 19.1%, 9% - 24%, and 0% - 43%, respectively (20). In previous studies from Iran, the antibiotic susceptibility profile has been different and it has been totally shown that resistance to erythromycin, cefotaxime, cotrimoxazole, chloramphenicol, and penicillin is increasing (21, 22). In the present study, most of MDR cases were resistant to cotrimoxazol, erythromycin, and tetracycline. Because of high resistance

to penicillin and more spread of MDR strains, misidentification of pneumococci leads to the increase in false report of non-susceptible isolates (15).

5.1. Conclusions

The *lytB* is similar to *lytA* in sensitivity for diagnosis of *S. pneumoniae* in isolates and clinical samples based on both molecular methods. The results confirmed the applicability of real time PCR based on *lytB* genes for detection of *S. pneumoniae*.

Acknowledgments

This research was supported by Tehran University of Medical Sciences, Tehran, Iran (Grant No.26772). We thank the individuals and organizations who participated in this study.

Footnotes

Authors' Contribution: Mohammad Azarsa: manuscript preparation and performing experimental procedures; Seyed Alireza Salami; design of real-time PCR assay; Mohammad Reza Pourmand: study concept and design, development of the study; Abbas Rahimi Forushani: participation in the acquisition of data and statistical analysis; Hamid Kazemian: manuscript revision. All the authors read and approved the final manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest.

Financial Disclosure: There was no financial disclosure to report.

Funding/Support: This research was supported by Tehran University of Medical Sciences, Tehran, Iran (grant No.26772)

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