

Evaluation of Prevalence of *S. pneumoniae* pharyngeal carriers under 5 years of age by *lytA* gene detection

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ABSTRACT

Background and Objective: *Streptococcus pneumoniae* is one of the leading causes of death among children worldwide. Nasopharyngeal colonization in children can spread pneumococcal infections in the community. This study aimed to evaluate the prevalence of *S. pneumoniae* strains isolated from healthy pharyngeal carriers less than 5 years of age.

Methods: This cross-sectional descriptive study was performed on 150 children under 5 years old in the city of Shiraz. After nasopharyngeal swab sampling, the samples were cultured on blood agar containing 5% sheep blood. The cultures were incubated at 37 °C for 24 h. Primary identification was carried out using optochin sensitivity testing, bile solubility testing and gram staining. Molecular identification of *S. pneumoniae* strains was done using *lytA* gene-specific primers.

Results: Of the 150 samples collected from healthy children, 24.67% were pharyngeal carriers of *S. pneumoniae*. The highest frequency of pneumococcal strains was related to male carriers (n= 22, 59.46%) and the children aged 1-2 years (n=11, 29.73%). The results showed no significant association between the prevalence of pharyngeal carriage and gender or age.

Conclusion: Given the increasing rate of pharyngeal carriage of *S. pneumoniae* in children as a risk factor for respiratory tract infections, there is a need for further monitoring of the circulating serotypes and investigation of antibiotic-resistance mechanisms.

Keywords: *Streptococcus Pneumoniae*, Pharyngeal Carriers, *lytA*.

INTRODUCTION

Streptococcus pneumoniae is a highly prevalent gram-positive microorganism. This bacterium could be found as an asymptotic part of normal nasopharyngeal bacterial flora in individuals, especially children. This phenomenon is apparently not dangerous for children, but the microorganism is able to migrate to other places such as the ears, lungs, bloodstream and cerebrospinal fluid under certain conditions and cause various effects on tissues (1). Thus, the pharyngeal carriers have the potential to develop acute pneumococcal infections such as pneumonia, meningitis, sinusitis and otitis media. According to the World Health Organization, one million deaths in children less than 5 years each year are associated with *S. pneumoniae* (2). Children carriers as the reservoir of this pathogen, also have a significant role in horizontal transmission of pneumococcal infections (3). Strains of *S. pneumoniae* in nasopharynx of healthy children are a reflection of the strains circulating in the community. Therefore, the investigation of this phenomenon can reveal useful information on policy-making for prevention and treatment of pneumococcal infections (4, 5). Optochin sensitivity is the most important and sometimes the only test used in most laboratories for detection of *S. pneumoniae*, and its differentiation from *Streptococcus viridans*. However, optochin-resistant strains have been reported in Finland (6), Spain (7) America (8), Portugal (9) and Argentina (10) from 1987 onwards, which have made difficult the detection of pneumococci. In addition to optochin sensitive test, bile solubility test is used for detection of the bacterium, but it may not always work (11-13). To overcome the challenges raised about the optochin sensitivity and bile solubility tests, polymerase chain reaction (PCR) method is used for accurate identification of *S. pneumoniae* and its virulence factors in clinical samples. It has been shown that PCR using *lytA* gene is much more specific than other virulent genes such as *ply* and *psaA*. Detection of the *lytA* gene is one of the best methods of *S. pneumoniae* identification. This gene encodes autolysin N-acetylmuramoyl-L-alanine amidase in *S. pneumoniae*. The *lytA* gene has an important role in virulence of the bacterium and the potential to be used in

vaccine production. The enzyme is within the cell envelope and involved in various physiological activities such as cell growth and cell detachment. N-terminal domain is responsible for the catalytic role of this enzyme. In addition, C-terminal composed of six repeating units, acts as a small tail that serves as the enzyme's choline-binding arm in the bacterial wall. Moreover, this gene is specific for *S. pneumoniae* and does not exist in *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus salivarius*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (14-16). This study aimed at molecular evaluation of prevalence of *S. pneumoniae* strains isolated from pharyngeal carriers less than 5 years of age using PCR and the *lytA* gene.

MATERIAL AND METHODS

This cross-sectional descriptive study was conducted on 150 randomly selected children aged < 5 years (age range 0-5 years) referred for medical care in city of Shiraz. Throat swab samples were obtained carefully and without touching the saliva and roof of the mouth. For isolation of *S. pneumoniae*, the samples were cultured on blood agar medium containing 5% sheep blood. The cultures were incubated at 37 °C for 24 hours. Preliminary identification of grown colonies was done based on properties of the colony, type of hemolysis, gram staining, optochin test and bile solubility test. A liter of the blood agar medium (Merck) contained 2 g yeast extract, 16 g peptone, 0.5 g glucose, 7 g sodium chloride and 17 g agar. This suspension was autoclaved at 121 °C and 15 pounds of pressure for 15 min. Temperature of the solution was raised to 45-50 °C, and 5% defibrinated sheep blood was added. After preparation and sterilization, the medium was divided into sterile plates under the hood and then kept in the refrigerator. In addition, disk diffusion method and Mueller Hinton culture medium containing 5% defibrinated blood were used for measuring sensitivity to optochin. In this study, the bacterial DNA was extracted via the boiling method. For this purpose, an amount of each sample was solved in microtubes containing 100 µl of distilled water. The samples were boiled for 10 min

at 100 °C. The microtubes were then centrifuged for 5 min at 13000 rpm. After extraction of the DNA from the supernatant, 2 µl of each sample was electrophoresed on agarose gel and then examined with UV light to control quality of the DNA. Finally, the extracted DNA was stored at -20 °C. In order to verify accuracy of the isolates, *lytA* gene amplification was done using the following specific primers *lytA*-F (5'-CAA CCG TAC AGA ATG AAG CGG -3 ') and *lytA*-R (5'-TTA TTC GTG CAA TAC TCG TGC G -3 ') (17). The primers were obtained from CinnaGen Co. (Tehran, Iran). PCR was carried out using a thermocycler (Biomedica) with initial denaturation at 95 °C for 5 minutes (hot start), 30 cycles of 94 °C for one minute (denaturation), 58 °C for one minute (annealing) and 72 °C for one minute (extension) and 72 °C for 5 minutes. PCR solution (25 µl) contained 1 µl of DNA template, 2.5 µl 10X buffer, 1 µl of each primer (10 mM), 1 µl MgCl₂ (50 mM), 0.5 µl dNTPs (10 mM), 0.25 µl Taq polymerase (5U/µl) and 17.75 µl distilled water. PCR products were studied on 1% agarose gel containing ethidium bromide, and later examined by a transilluminator device. In this study, *S. pneumoniae* ATCC49619 (standard strain) was used as the positive control. Finally, the results were analyzed in SPSS (version 15) using Fisher's exact test. P- values less than 0.05 were considered

statistically significant.

RESULTS

Among 150 healthy children under the age of 5 years, 98 (65.33%) were male and 52 (34.67%) were female. After performing the biochemical tests and detection of the *lytA* gene, 37 children (24.67%) were identified as *S. pneumoniae* pharyngeal carrier. The mentioned colonies were gram-positive, optochin-sensitive, bile-soluble and alpha-hemolytic. In addition, amplification of the 295 bp sequence using *lytA*-F and *lytA*-R primers indicated the presence of *S. pneumoniae* (Figure 1). According to Table (2), frequency distribution and ratio of pharyngeal carriers were investigated based on gender and age. Of 37 pharyngeal carriers, 22 (59.46%) were male and 15 (40.54%) were female. Fisher's exact test showed no significant association between the frequency of *S. pneumoniae* carriage and gender of study subjects (P=0.318). The pharyngeal carriers were divided into five age groups. Most carriers were aged 1-2 years with frequency ratio of 11 (29.73%). The lowest frequency of carriage was observed in the 4-5 year children with frequency ratio of 4 (10.81%). There was no significant association between the frequency of pharyngeal carriage and age of the participants in this study (P=0.151).

Figure 1- 295 bp fragments resulted from *lytA* gene amplification

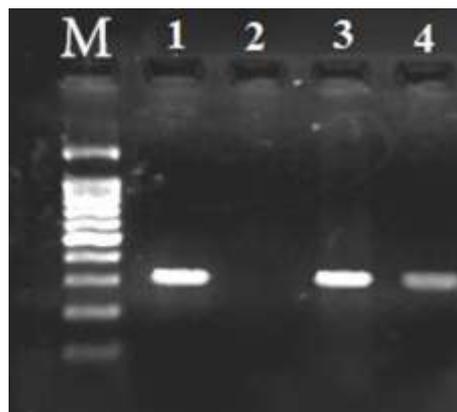


Table1- The frequency of pharyngeal carriers of *S. pneumoniae* in healthy children based on gender and age (n=37)

Age group	Female carriers Number (%)	Male carriers Number (%)	<i>lytA</i> -positive isolates Number (%)
Less than 1year	2(5.41)	5(13.51)	7(18.92)
1-2 years	6(16.22)	5(13.51)	11(29.73)
2-3 years	3(8.11)	6(16.22)	9(24.32)
3-4 years	3(8.11)	3(8.11)	6(16.22)
4-5 years	1(2.70)	3(8.11)	4(10.81)
Total	15(40.54)	22(59.46)	37(100)

DISCUSSION

Since pharyngeal carriers of *S. pneumoniae* possess all the factors necessary to develop an acute and invasive infection, various studies have been conducted on this issue in different parts of the world (2). The prevalence of *S. pneumoniae* pharyngeal carriage varies in different populations. In this study, 37 children (24.67%) aged less than 5 years were pharyngeal carriers. The highest frequency of carriage was related to the 1-2 years age group. In a study in Tehran, 1300 healthy children studying in different schools were randomly selected, among which 44.1% were pharyngeal carriers of *S. pneumoniae* with mean age of 5-7 years (18). Moreover, Ghaemi et al. investigated the prevalence of pneumococcal pharyngeal carriers in healthy primary school children in Gorgan.

Among 1268 healthy children studied, 10.9% were *S. pneumoniae* carriers (19). Study of Bakhshae et al. reported that 8.78% of children under the age of six in kindergartens of Mashhad are healthy *S. pneumoniae* nasopharyngeal carriers (20). This issue has been also considered in other countries. In a study on 291 healthy under-five children in the USA, 47 (16.2%) were pharyngeal carriers of *S. pneumoniae* (21). In Taiwan, a study on 2905 samples from children aged 2 months to 7 years old in different kindergartens reported that 21% were healthy carriers of *S. pneumoniae* (22). In a study in the Philippines, the frequency of *S. pneumoniae* pharyngeal carriage was 47.5% in children aged less than five years with the highest frequency observed in the 3-12 months age group (23). In the studies conducted in Italy and Belgium, the frequency of healthy pharyngeal carriers was reported as 8.6% and 21%, respectively. Most

carriers in the mentioned studies belonged to the 3-36 months age group (5, 24). Based on the results of the present study and previous studies, it can be concluded that the prevalence of *S. pneumoniae* pharyngeal carriage is different in healthy children aged less than 5 years and other age groups. The differences in the frequency of *S. pneumoniae* carriage in different countries could be due to different climate conditions, population density and geographical location.

This bacterium colonizes in the upper respiratory system of healthy children. The presence of this microorganism in the throat plays an important role in its spread in family, school and daycare centers. Attendance at daycare centers, early age, antibiotic use, and history of infection or underlying disease are some of the risk factors affecting the carriage of this microorganism (25). Therefore, further studies are recommended on the identification of serotypes circulating in the community with the aim to develop vaccines for prevention. Moreover, antibiotic resistance patterns should be investigated in order to determine optimal treatment.

CONCLUSION

According to the results obtained in this study, the prevalence of *S. pneumoniae* pharyngeal carriage in healthy children under 5 years is increasing in the city of Shiraz. Since these individuals have the potential to develop acute pneumococcal infections, and considering the climatic conditions of Shiraz, increasing spread of pharyngeal carriage, especially in daycare centers, kindergartens

and schools is expected. Therefore, policies should be made and implemented to control this issue in this region.

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CONFLICT OF INTEREST

We have no conflict of interest to declare.

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