Iranian Journal of Microbiology
Volume 7 Number 3 (June 2015) 156-160

ORIGINAL ARTICLE

Detection of *lytA*, *pspC*, and *rrgA* genes in *Streptococcus pneumoniae* isolated from healthy children

Tahereh Gholamhosseini-Moghaddam[1], Mehrnaz Rad [1*], Seyed Fazlollah Mousavi[2*], Kiarash Ghazvini[3]

[1]Department of Pathobiology, School of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
[2]Department of Bacteriology and Microbiology Research Center, Pasteur Institute of Iran, Tehran, Iran.
[3]Antimicrobial Resistance Research Center, Faculty of Madicine, Mashhad University of Medical Sciences, Mashhad, Iran.

Received: December 2014**, Accepted:** May 2015

ABSTRACT

Background and Objectives: Many surface proteins are implicated in nasopharyngeal colonization and pathogenesis of *Streptococcus pneumoniae*. Some of these factors are candidate antigens for protein based vaccines. New vaccine designs focus on the surface proteins (e. g., *pspA* and *pspC*) and also cytolysin, and pneumolysin. In this study, 3 key virulence genes, *lytA*, *pspC*, and *rrgA*, which encoded surface proteins, were detected among *S. pneumoniae* isolates.
Materials and Methods: A total of 260 nasopharyngeal swabs were collected from healthy children under 6 years old attending day care centers in Mashhad, Iran. Isolates of *S. pneumoniae* were confirmed by optochin susceptibility and colony appearance and also by PCR for *cpsA* gene. The presence of *lytA*, *pspC*, and *rrgA* genes were also detected by PCR.
Results: A total of 59 isolates were confirmed as *S. pneumoniae*. Among these isolates, 50 (84.74%), 19 (32.20%), and 2 (3.38%) were positive for *lytA*, *rrgA*, and *pspC* genes respectively. The presence of these genes among *S.pneumoniae* isolates were as follows: 1) *rrgA*, *lytA*, *pspC* (1 isolate), 2) *rrgA*, *lytA*(17isolates), 3) *pspC* (2 isolate), 4) *lytA* (50 isolates).
Conclusion: *cpsA* gene was specific for detection of *S. pneumoniae* isolates which were colonized in nasopharynx. The lyt*A* gene was the most frequent gene among the *S. pneumoniae* isolates, and combination of *rrgA*, *lytA* was the most observed pattern. Thus, it is important for future monitoring of vaccine formulation in our country.
Keywords: *Streptococcus pneumoniae*, *lytA*, *pspC*, *rrgA*, children.

INTRODUCTION

Streptococcus pneumoniae remains a major cause of childhood morbidity and mortality worldwide, particularlyin lower income countries. Pneumococcal diseases are the leading source of vaccine preventable deaths, mostly due to community-acquired pneumonia (CAP), accounting for approximately 11% of all deaths in children under 5 years old (1). Colonization of the nasopharynx is a necessary step along the path to pneumococcal disease (PD) (2). Pneumococcal conjugate vaccines (PCV) reduce nasopharyngeal carriage of serotypes which included in the vaccine by conferring capsular-specific immunity. Experience from countries where conjugate vaccines have been introduced has shown rapid and sustained carriage reduction of vaccine serotypes (VT) following vaccination (3). Adhesins are essential for pneumococcal colonization and pathogenesis (4).

*Corresponding author: Mehrnaz Rad, Seyed Fazlollah-Mousavi
E-mail: rad@um.ac.ir, sadatff@yahoo.com

The microorganism produces a plethora of virulence factors, including the polysaccharide capsule, several surface-located proteins, and the toxin pneumolysin (5, 6). The polysaccharide capsule is highly efficient in protecting the bacteria from opsonophagocytosis (7). Surface proteins of *S. pneumoniae* (*pneumococcus*) have been investigated for their role in pneumococcal pathogenicity and as candidate antigens for protein based vaccines (8). Among the surface-associated proteins, the pneumococcal surface protein A (PspA) andC (PspC) are the best characterised choline-binding proteins (6, 9). Pneumolysin is a cytoplasmic toxin released by autolysis of the cell and it is a very important virulence factor with multiple effects. The major autolytic enzyme of the pneumococcus is LytA (Nacetylmuramoyl-L-alanine-amidase), which is responsible for the deoxycholate- and penicillin induced cell lysis in the stationary phase, having a great clinical importance (10). The *lytA*-encoded major autolysin of *S. pneumoniae* is a member of a widely distributed group of cell wall-degrading enzymes located in the cell envelope and postulated to play roles in avariety of physiological functions (11).

rrgA is a virulence factor in a murine lung infection model and has a varied distribution among serotypes of *S.pneumoniae* (12). *S. pneumoniae* adherence was significantly enhanced by expression of an extracellular pilus composed of three subunits, *RrgA, RrgB* and *RrgC* (13).

Some of the pneumococcal virulence factors are potential targets for protein- based pneumococcal vaccine production. Thus, in this study the presence of three genes were detected among the isolates of *S. pneumoniae.*

MATERIALS AND METHODS

Sampling. A total of 260 samples from nasopharynx of healthy children under 6 years old were taken carefully and transferred under cold condition to the laboratory. These samples were collected from children attending day care centers in different geographical areas of Mashhad, Iran.

Isolation. All nasopharynx samples were plated on blood agar and chocolate agar plates. Both plates were incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours. The plates were examined and α-haemolytic colonies suspected to be *Streptococcus pneumoniae* were confirmed by optochin test and PCR for *cpsA* gene as described earlier (14). The optochin-susceptibility test was performed using a 6.5 mm diameter disc containing 5 mg optochin (Oxoeid) in an atmosphere of 5% CO_2. A zone of inhibition of at least 14 mm diameter constituted a positive result.

DNA extraction. The genomic DNA of the bacterial isolates were extracted by DNAase Tissue kit (KIAGEN, Tehran, Iran).

Detection of virulence genes. The presence of *rrgA, pspC* and *lytA* genes were detected among confirmed *S. pneumoniae* isolates by three single PCR assays. The oligonucleotide sequences of primers used in this study are listed in Table 1.

Table 1. Oligonucleotides which were used as primers to amplify particular sequences of *S. pneumoniae*

Gene	Primers	Size of amplicon(bp)	Ref.
lytA	F: 5'-CAA CCG TAC AGA ATG AAG CGG-3' R: 5'-TTA TTC GTG CAA TAC TCG TGC G-3'	319bp	15
pspC	F: 5'- AAGATGAAGATCGCCTACGAACAC-3' R: 5'- AATGAGAAACGAATCCTTAGCAATG-3'	1000-1200bp	16
rrgA	F: 5'-.CACTTTTATACGCTTTTGCTA-3 R: 5-'TAATACGACTCACTATAGGTGCCATCCG-TATTGTTTTTC-3'	373bp	17

PCR assays. Amplification conditions for *LytA* and *pspC* genes were: 94° C for 2 min, 25cycles of 94° C for 10 s, 58° C for 15 s, and 72°C for1 min, followed by a final extension at 72 °C for 5 min. *S. pneumoniae* ATCC 3340) was used as positive control.

Amplification conditions for *rrgA* gene were: 95°C for 2 min, 25cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 90 s, followed by a final extension step at 72°C for 5 min. The amplicons were observed under UV light after electrophoresis.

Sequence analysis. One amplicon from *S. pneumoniae* with the gene *rrgA* was sequenced by Macrogen company (South Korea). Sequences were examined for identity with published sequence data from National Center for Biotechnology Information (NCBI).

RESULTS

Among 260 nasopharanx samples from healthy children under 6 years, 59 isolates were confirmed as *S. pneumoniae*. All isolates were susceptible to optochin and were positive for *cpsA* gene.

Distribution of the *lytA*, *rrgA* and *pspC* genes among isolates of *S.pneumoniae* were determined. Fifty isolates (84.74%), were positive for *lytA*. *rrgA* and *pspC* were also found in17 (28.81%), and 2 (3.38%) isolates respectively. Five patterns of these genes were seen among *S. pneumoniae* isolates: *lytA* (n=33, 55.93%), *rrgA* (n=2, isolates, 3.38%), *pspC* (n=1 1.69%), *lytA*+*rrgA* (n=16, 27.11%) and *lytA*+ *rrgA*+ *pspC* (n=1, 1.69%).

Sequence analysis. The amplicon represented expected sequences with more than 90% identity with published data from NCBI (GenBank: EF 560634.1). This was used as a positive control for *rrgA* gene in PCR reactions.

DISCUSSION

Pneumococcus is a frequent colonizer of the nasopharynx in children. It remains unclear, however, why some children develop invasive disease, whereas in the majority of cases, colonization remains asymptomatic, a combination of bacterial virulence and host factors may be responsible (18). We studied the *S. pneumoniae* isolates giving special reference to identification and distribution of virulence markers such as autolysin among the isolates from nasopharynx. This exercise may help in understanding the factors contributing to the pathogenicity of *S. pneumoniae*. Further, there are numerous reports giving us increasing evidences on the role of *lytA* in pneumococcal pathogenesis suggesting that this might be more appropriate as vaccine antigen against *S. pneumoniae* infections.

The *cpsA* was used as a novel genetic marker specific for identification of *S. pneumoniae* and to differentiate it from the closely viridans group streptococci as well as other pneumococcus-like streptococci such as *S. pseudopneumoniae* (19). Many virulence genes contribute to the colonization of *S. pneumoniae*; however, our study only demonstrates this for *pspC*, *rrgA* and *lytA* genes. Thus, synergistic effect and correlation of these virulence factors is necessary for our future work.

Our results showed that most of the isolates had *lytA* gene (84.74%) which has an important role in colonization. Only 9 out of 59 isolates were negative for the presence of this gene. This observation is in concordance with previous report which was showed that all of the unencapsulated isolates of *S. pneumoniae* were negative for *lytA* and *psaA* by PCR. Detection of *lytA* and *psaA* in six encapsulated isolates for which a serotype could be determined was negative (9). False negative results were obtained by the PCR assays for these two genes may be due to mutations or sequence variation. On the other hand, *lytA* is essentially a rather conserved gene displaying limited genetic variation (11).

In the study of Whatmore, 33 out of 62 isolates of *S. pneumoniae* were selected to represent a diverse range in terms of serotype, clinical association, and time and place of isolation.

Autolysin which was found in all strains, might appear to be a suitable target virulence, and apparently highly conserved, for inclusion in a potential vaccine (11).

The pneumococcal protein Lyt A is the major autolysin of *S. pneumoniae*, it has an important function in pathogenesis by releasing pneumolysin and plays a fundamental biological role in bacterial lysis after exposure to certain antibiotics (19). It has been reported that the *lytA* gene has higher specificity than the *pspC* for identification of *S. pneumoniae* (15, 20). In our study, only 2 out of 59 isolates of *S. pneumoniae* (3.38%) had *pspC* gene. Given the high

sequence diversity of *pspC*, it is unlikely that PspC alone can be a vaccine antigen to provide protection from across different pneumococcal strains (16).

It was showed that RrgA is central in pilus-mediated adherence and disease, even in the absence of polymeric pilus production (13). However, it has been demonstrated that numerous protein virulence factors are involved in the pathogenesis of pneumococcal disease (21). Hence, new vaccine designs are focused on the surface proteins (e. g., PspA and PspC), cytolysin, and pneumolysin (22).

CONCLUSION

We concluded that the gene *cpsA* was specific and highly conserved among *S. pneumoniae* isolates which were colonized in nasopharynx. On the other hand, we showed that *lytA* gene was the most frequent genes among the *S. pneumoniae* isolates, and combination of *rrgA, lytA* was the most observed pattern.Thus this should allow for appropriate screening of adhesin-based vaccines to prevent infections by streptococci.

ACKNOWLEDGEMENT

This project was supported by research grant (Grant No 2829) of Ferdowsi University of Mashhad. The authors wish to thank of Mr. Saman Nobari for his technical assistance.

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