Original Article

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Demonstration of Virulence Factors in *Streptococcus Pneumoniae* Isolates from a Tertiary Hospital in South India

Microbiology Section

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ABSTRACT

Introduction: *Streptococcus pneumoniae* (pneumococci) colonizes the nasopharynx and 94 different serotypes have been characterized on the basis of the capsular polysaccharides. Nasopharyngeal colonization is documented to be present in children more, than that seen in adults. Pneumococci cause wide range of infections with marked morbidity among children, elderly and immuno-compromised patients. The pathogenesis may be attributed to the virulence factors such as pneumolysin and autolysin.

Aim: The aim of this study was to demonstrate virulence genes, pneumolysin (*ply*) and autolysin (*lytA*) in pneumococci isolated from clinical samples and nasopharyngeal carriers by Polymerase Chain Reaction (PCR).

Materials and Methods: Total 15,473 clinical samples of sputum, broncho-alveolar lavage, tracheal aspirate, cerebro-spinal fluid, blood, ascitic fluid, throat swab, eye discharge, ear swab and pleural fluid from infected patients, including nasopharyngeal carriers screened for pneumococci in 600 healthy school children were included in the study. Blood and CSF isolates were categorized as invasive and those isolates obtained from other clinical sites as non-invasive, respectively. Standard methods for characterisation of *Streptococcus pneumoniae* such as Gram stain, Optochin sensitivity, bile solubility, and inulin fermentation was done, with confirmation by automated Vitek 2. *Streptococcus pneumoniae* isolated from positive samples were subjected to molecular detection of virulence genes *lytA* and *ply* coding for autolysin and pneumolysin respectively.

Results: In all 121 *Streptococcus pneumoniae* positive isolates were isolated from clinical specimens including carriers with isolation rate of 0.81%. Seventy four pneumococci isolates were randomly selected which were representative of invasive, non-invasive and carrier isolates. They were screened by PCR for *lytA* and *ply* genes using appropriate primers. All isolates tested were positive for autolysin (*lytA*) and 97.2% including the 15 invasive isolates were positive for pneumolysin (*ply*).

Conclusion: This study concludes that detection of virulence genes for pneumococci in clinical samples confirms the patho-physiologic consequences of pneumococcal infections.

Keywords: Autolysin, Pneumolysin, Pneumococci, Virulence genes

INTRODUCTION

Streptococcus pneumoniae inhabit the upper respiratory tract and causes invasive infections such as meningitis, bacteremia and broncho-pulmonary infections which is enhanced by virulence factors such as the capsule, *pneumococcal* surface protein A and C, autolysin and pneumolysin [1-5]. Autolysin which is encoded by *lytA*, disrupts the bacterial cell wall at the site of attachment of stem proteins, and releases peptidoglycan components thereby activating the production of complement. Pneumolysin produced by all invasive strains is a species-specific 53 kilo Dalton, thiol activated virulence factor that inserts into the lipid bilayer of cell membranes with cytolysin and complement-binding properties and is encoded by *ply* gene [6,7]. Evaluation of diagnostic usefulness of pneumolysin and its corresponding antibody detection in clinical specimens is beginning to receive attention [8-11].

The objective of this study was to demonstrate genes of virulence factors such as pneumolysin and autolysin in pneumococcal isolates from various clinical samples and nasopharyngeal carriers by molecular techniques and to analyze the potential of candidate pneumolysin vaccines in preventing invasive pneumococcal infections.

MATERIALS AND METHODS

This prospective study was done in the Department of Microbiology at PSG Hospitals, Coimbatore, India for a period of 27 months starting from 2011 till 2013 after obtaining Ethics Committee's approval. Informed consent and Clinical samples such as sputum, broncho-alveolar lavage, tracheal aspirate, cerebro-spinal fluid, blood, ascitic fluid, eye discharge, ear swab and pleural fluid were taken from infected patients. Nasopharyngeal swabs from 600 healthy school children aged 12 -14 years with consent from guardian/parentwere taken. Demographic and clinical

details of patients such as age, gender, clinical diagnosis, site of clinical sample and history of prior antibiotic intake were recorded.

Invasive isolates were pneumococcal samples isolated from blood and CSF, and the rest were categorized as non-invasive isolates. Recovery of *Streptococcus pneumoniae* was obtained by inoculating on 5% sheep blood agar and chocolate agar, followed by overnight incubation in candle jar with 5% CO_2. Gentamycin blood agar (2.5 mg/l) was used in sputum samples to improve the yield of pneumococci. Those isolates which grew alpha hemolytic colonies were characterized as pneumococci by Gram stain showing lanceolate diplococci, capsule staining by India ink, carrom-coin appearance on blood agar, optochin sensitivity with zone of inhibition greater than 14 mm, bile solubility (tube method) containing sodium deoxycholate, fermentation of inulin and identification by automated system Vitek-2. Isolates were stored at -20°C in medium containing glycerol and skimmed milk powder. Revival of isolates and DNA extraction was done followed by detection of virulence genes *lytA* and *ply* by PCR.

School children aged less than 12 years and more than 14 years, and clinical isolates other than *Streptococcus pneumoniae* were excluded from the study.

Out of 121 isolates which were identified and confirmed as *Streptococcus pneumoniae*, 74 pneumococcal isolates which were representative of the three categories (invasive, non-invasive and carrier) were subjected to molecular detection of virulence genes (15 invasive isolates, 50 non-invasive and 9 carriers). Pneumococcal colonies in 5 ml of brain heart infusion broth were centrifuged at 2000 rpm for 10 minutes. Sediment was used for DNA extraction after discarding the supernatant fluid. Pre-extraction and extraction steps required to extract DNA were done as per manufacturer's instructions using the QIAamp 50 Mini Amp kit from QIAGEN. Eluted genomic DNA was stored at 4°C for further analysis and at -20°C for long term storage. We detected autolysin gene and pneumolysin by amplifying the 319bp fragment encoded in *lytA* gene and 348bp fragment present in *ply* gene respectively by obtaining the required primer sequences from study by Kanungo R et al., [3]. Reagents required for PCR and the primers were procured from SIGMA-ALDRICH.

lytA gene:

Forward primer: 5'-CAACCGTACAGAATGAAGCGG-3'

Reverse primer: 5'-TTATTCGTGCAATACTCGTGCG-3'

Ply gene:

Forward primer: 5'-ATTTCTGTAACAGCTACCAACGA-3'

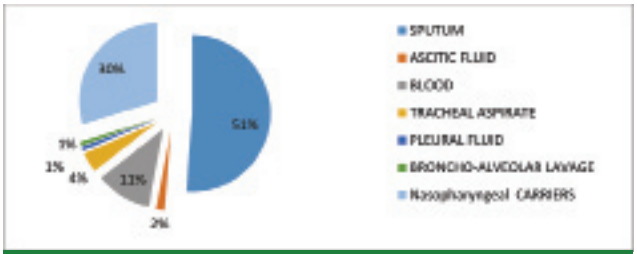
Reverse primer: 5'- GAATTCCCTGTCTTTTCAAAGTC- 3'

PCR amplification was done using Eppendorf 950000040 Mastercycler personal thermal cycler with heated Lid. PCR reaction mixture for each pneumococcal isolate contained 12.5 µl master mix, 1 µl each of all 4 primers, 3.5 µl of PCR water and 5 µl of template DNA. The thermocycler settings were initial denaturation at 94°C for 5 minutes, 30 cycles of PCR, with each cycle consisting of 30 seconds at 94°C, 30 seconds at 53°C and 30 seconds at 72°C with final extension at 72°C for 10 minutes. PCR products were analysed using 1.8% agarose gel electrophoresis, with ethidium bromide staining and the bands were visualized under UV illumination.

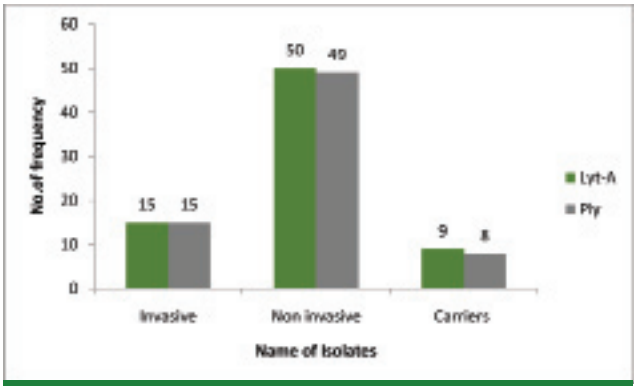
RESULTS

Totally 121 positive *Streptococcus pneumoniae* were isolated from 15,473 clinical specimens (including 600 nasopharyngeal carriers) with an isolation rate of 0.81%. Analysis of data showed more than 50% were isolated from sputum specimens, followed by 30% in carriers and 11% in invasive isolates [Table/Fig-1].

Out of 74 *Streptococcus pneumoniae* isolates subjected to molecular detection of *lytA* and *ply* gene, *lytA* gene was found in all of them but 2 non-invasive isolates including the carrier isolate which was negative for *ply* gene [Table/Fig-2-4].



[Table/Fig-1]: Distribution of *Streptococcus pneumoniae* isolated from various specimens.

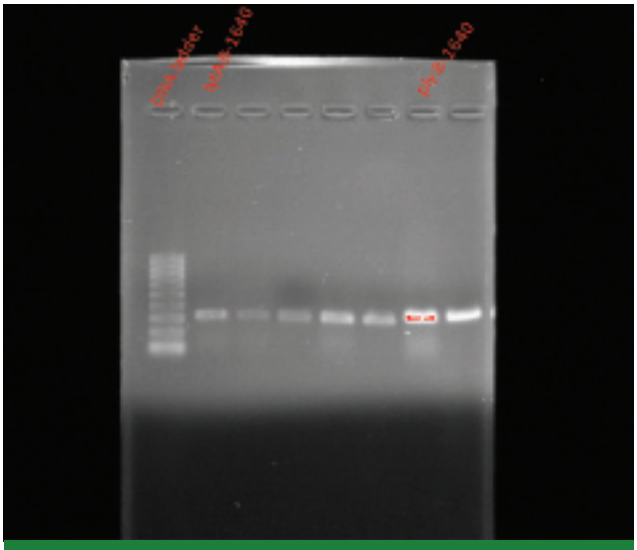


[Table/Fig-2]: Showing data on the number of isolates with detection of *lytA* and *ply* gene.

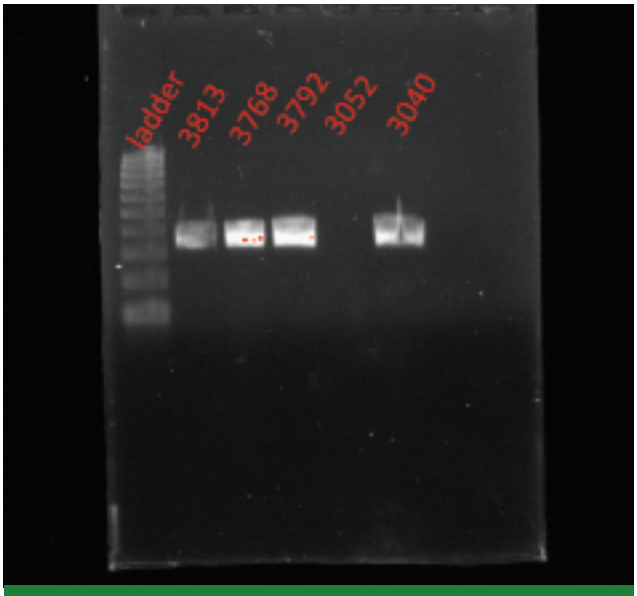
STATISTICAL ANALYSIS

The minimum expected count is 8.38. Zero cells (0%) have expected count less than 5.

Comparing the different types of isolates with *lytA* and *ply*, with Chi-square value is 0.042 with p-value is 0.979. Hence,



[Table/Fig-3]: Bands showing both *lytA* (319bp) and *ply* gene (348bp) for a blood isolate.



[Table/Fig-4]: Bands showing absence of *ply* gene (348 bp) in 1 carrier isolate.

	Value	df	p-value (2-sided)
Pearson Chi-Square	0.042a	2	0.979 (NS)
Likelihood Ratio	0.042	2	0.979 (NS)
Linear-by-Linear Association	0.030	1	0.862 (NS)
No. of Valid Cases	146		

[Table/Fig-5]: Chi-Square Test.
a. 0 cells (.0%) have expected count less than 5.
NS=Non-significant

statistically there is no association relationship between them [Table/Fig-5].

DISCUSSION

About 97.2% of the isolates tested were positive for pneumolysin (*ply*) gene. Pneumolysin produced by all invasive strains, is a species-specific 53 kD, thiol activated virulence factor, interacting with cell wall lipid where membrane integrity is compromised due to pore formation. The significant patho-physiologic effects of pneumolysin are cytolysis and complement-binding property which warrants early detection and treatment. Sourav S et al., have done similar studies to detect genes coding for pneumolysin and autolysin in strains among Indian patients. Their study identified *lytA* in 23 out of 24 isolates tested whereas, only 17 isolates were positive for pneumolysin. They have concluded that pneumococci need autolysin which is an obligate need for its survival and pathogenesis , and pneumolysin is a critical component in causing invasive infections [6].

Since pneumolysin is a protein, it has been prudent to develop recombinant antigens, and the relevant monoclonal antibodies are being used in clinical detection. Recombinant candidate vaccines from PlyD1 have been reported to be adequately immunogenic and also safe [12,13].

Autolysin is an enzyme which destroys the cell wall, causing cell lysis and the subsequent release of pneumolysin that is not actively exported from the cell [14]. In a study by Whatmore AM et al., for *lytA* as a vaccine target, it has been postulated that *lytA* is a gene that is highly conserved, hence useful for identification [15]. In our study, detection of *lytA* gene encoding autolysin was seen in all the 74 isolates tested, thereby confirming identification of *Streptococcus pneumoniae*.

The clinical significance of our study reinforces the identification of virulence genes by molecular methods which authenticates the patho-physiologic consequences of pneumococci, and further research on utilizing pneumolysin in candidate vaccines is bound to give a ray of hope in mitigating the onslaught of deadly invasive pneumococcal infections.

LIMITATIONS

Only 74 of these fastidious isolates were subjected to molecular detection of virulence genes, since the others lost their viability during storage (-20°C in skimmed milk and glycerol) and could not be revived even after best efforts.

CONCLUSION

Routine laboratory markers to detect *Streptococcus pneumoniae* in clinical samples have their own shortcomings and have to be substantiated by detection of virulence genes *lytA* and *ply*, encoding for autolysin and pneumolysin which confirms the identification and patho-physiologic effects of *Streptococcus pneumoniae*. Candidate anti-

pneumolysin vaccines offer new promise in tackling invasive pneumococcal infections.

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