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Screening of Pneumococcal Pneumonia by Amplification of Pneumolysin Gene in Children Visiting Hospitals in Lahore, Pakistan

Mahjabeen Saleem*[1], Phd; Mamoona Naz[1], MSc; Asma Waris[1], PhD; Bushra Muneer[2], PhD; and Rukhshan Khurshid[3], PhD

1. Institute of Biochemistry and Biotechnology, University of the Punjab, Pakistan
2. Institute of Industrial Biotechnology, Govt. College University, Pakistan
3. Department of Biochemistry, Fatima Jinnah Medical College, Lahore, Pakistan

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Abstract

Objective: *Streptococcus pneumoniae* is a common worldwide potential pathogen causing pneumonia among children and the detection of pneumococcal infections by conventional culturing techniques is cumbersome. The present study describes a comparative analysis of sensitive nested-PCR and bacterial culture in pediatric patients with clinical and radiological indication of *S. pneumoniae* infection.

Methods: PCR was performed using outer primers to amplify a 348-bp region and inner primers a 208-bp region of the pneumolysin gene. For pneumolysin PCR assay, DNA from peripheral blood and middle ear fluid (MEF) samples was extracted by salting out method**.** The sensitivity of the assay was evaluated with about 0.06 pg of purified *S. pneumoniae* genomic DNA.

Findings: Among 90 MEF culture negative samples from acute otitis media pediatric patients, 8.8 % pneumolysin-PCR positivity was detected, demonstrating the sensitivity and reliability of PCR for rapid pneumonia evaluation. Binomial test of proportionality performed on (SPSS 17) gives $P<0.05$ indicating that PCR technique is statistically significant and sensitive in the diagnosis of *S. pneumoniae* infection.

Conclusion: The research work evaluated the effectiveness and efficacy of nested-PCR for detecting *S. pneumoniae* in pediatric patients with clinical and radiological confirmation of bacterial infection. This simplified method permitted quick selection of the patients and played a significant role in preliminary management of pneumococcal infections.

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Key Words: Streptococcus pneumoniae; PCR; Screening; Pneumolysin; Gene; Pneumococcal Infections

Introduction

The most common serious pneumococcal disease is pneumonia, an infection of the tissues of the lungs. Pneumococci may also cause infections in the ear (otitis media), para nasal sinuses (sinusitis), the tissues covering the brain and spinal cord (meningitis), and, less often, the heart valves, joints, and abdominal cavity. The capsular polysaccharide is the main virulence factor that protects pneumococci against the action of host defense mechanisms[1]. Pneumococcus is a leading pathogen, causing infections with high mortality and morbidity[2]. At least one million children die annually from pneumococcal diseases, and most of them are young children in developing countries[3]. The spectrum of pneumcoccal diseases differs in different age groups and

*** Corresponding Author;**
Address: University of the Punjab,Institute of Biochemistry and Biotechnology
E-mail: mahjabeensaleem1@hotmail.com

different populations[4]. Several risk factors for pneumococcal infections, such as age, race, immunodeficiency, other illness, socio-economic status, previous antibiotic therapy and day care attendance have been reported[5]. *Streptococcus pneumoniae* is facultative anaerobe, gram positive, capsulated organism that usually grows in pairs or short chain[6].

Molecular techniques have significantly improved the diagnostic yield and decreased the time for pathogen identification. These techniques are helpful in the determination of drug sensitivity and the understanding of transmission and outbreaks[7]. The effect of swab composition and compared the sensitivities of detection of *S. pneumoniae* in simulated and clinical respiratory specimens by culture and PCR-based methods have been evaluated[8].

This work aims to evaluate the sensitivity of pneumolysin-targeted nested-PCR for the detection of *S. pneumonae* and its comparison with routine clinical methods.

Subjects and Methods

Patients:

Pediatric patients with age 1-6 months (Group A) and up to 5 years (Group B) admitted to Mayo Hospital, Children Hospital and Services Hospital, Lahore, Pakistan with a clinical and radiological picture suggestive of pneumonia were enrolled in the study. They had fever of ≥39°C and a leukocyte (WBC) count of ≥15×10^9/liter suspected to have invasive bacterial infection. In Group A, 98 patients and 145 in Group B fulfilled the criteria for suspected invasive pneumococcal infection. Blood samples from 60 healthy persons were also collected to test the specificity of the PCR assay. Blood samples were collected before and after antibiotic treatment.

In other study, middle ear fluid (MEF) samples were taken from the acute otitis media (AOM) patients from the ENT department of Mayo hospital. AOM was defined by the otoscopic examination suggesting MEF and at least one of the symptoms: rubbing of the ear, runny nose, cough, ear pain or temperature of at least 38^{o}C.

Peripheral blood and MEF sample collection:

The blood samples were collected in tubes containing EDTA from each patient during his/her initial visit. Middle ear fluid samples were taken from the acute otitis media patients in sterilized cotton swabs. Cotton tipped swab was inserted into the ear, aspirated and the secretion was rinsed out with 0.5 ml of phosphate buffered saline. MEF bacterial culture was inoculated on selective blood agar media containing 5 µg/ml gentamicin and incubated at 37°C for 72 h in a 5% CO_2 atmosphere.

DNA extraction from peripheral blood:

DNA was isolated from blood samples according to the salting out method[9]. Blood samples (300 µl) were resuspended in a 1.5 ml microfuge tube containing 900 µl RBC lysis solution (10 mM Tris-HCl, 400 mM NaCl and 2 mM Na_2 EDTA, pH 8.2). The cell suspension was digested with 0.2 ml of 10% SDS and 0.5 ml of a proteinase K solution (1 mg proteinase K in 1% SDS and 2 mM Na_2 EDTA) at 37^{o}C for overnight. One ml of 6 M NaCl was added and shaken vigorously for 15 sec, followed by centrifugation at 13,000× g for 15 min.

The supernatant containing DNA was transferred to another tube, added double volume of absolute ethanol and inverted several times until the DNA precipitated. Supernatant after centrifugation was decanted and drained briefly on a clean absorbent paper. DNA was washed three times with 70% ethanol and centrifuged at 13,000× g for 1 min. DNA pellet was dissolved in 25 µl of autoclaved distilled water.

DNA extraction from MEF:

Pneumococcal cells from MEF samples were suspended in the phosphate buffer saline by rubbing each swab against the wall of tube and centrifuged at 12,000× g for 10 min. The pellet was suspended in a buffer containing 10 mM Tris, 0.14 M NaCl, 0.1 M sodium citrate and 10 mM Na_2 EDTA and incubated at 80^{o}C for 10 min. DNA was extracted by the method as described above.

Pneumolysin PCR amplification:

The selection of the primers was based on the published sequence of the pneumolysin gene[10].

PCR amplification of a 50 µl reaction mixture containing pneumolysin specific primers was performed in a programmable thermal cycler (Eppendorf). The outer primers (5'-ATTTCTGTAACAGCTACCAACGA-3') and (5'-GAATTCCCTGTCTTTTCAAAGTC-3') amplified a 348-bp region of the pneumolysin gene, and the inner primers (5'-CCCACTTCTTCTTGCGGTTGA-3') and (5'-TGAGCCGTTATTTTTTCATACTG-3') amplified 208-bp region. The reaction mixture contained 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM deoxyribonucleotides, 50 pmol of primers, 1U of *Taq* DNA polymerase (Fermantas) and 2 µl of DNA (~250 ng) extracted from whole blood and MEF fractions.

The amplifications were repeated 35 times as follows: denaturation at 94°C for 45 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec. After 35 cycles, the samples were incubated for a further 6 min at 72°C and stored at 4°C until analyzed. Amplifications were carried out as for the first-round PCR, autoclaved double distilled water and DNA of healthy volunteers used as negative control. PCR amplified products mixed with 6× bromophenol blue loading dye were run in a 2% agarose gel, stained with ethidium bromide and visualized under UV light Gel Documentation system.

Statistical Analysis:

SPSS version 17.0 was used to perform binomial test to demonstrate the statistical significance of PCR for diagnosis of pneumococcal infections.

Findings

Patient characteristics:

The age of the children was 1-6 months (Group A) and above one year (Group B). The clinical diagnosis and numbers of pediatric patients in the Group A, fulfilling the study inclusion criteria (n=98) were as follows: pneumonia 44; acute respiratory tract infection 30; pneumococcemia 24. The study inclusion criteria in Group B (n=145) were as follows: pneumonia 80; fever without infection focus 8; acute respiratory tract infection 28; pneumococcemia 22; gastroenteristis 3 and acute tonsillitis 4. Sixty healthy person samples were used as control. In Group A, the highest WBC count values were 23 x 10^9 to 15 x 10^9/L and the highest body temperatures 45 ± 1.1 and 38.8 ± 1.2°C, respectively. In Group B, the highest WBC count values were 20.2×10^9 to 15.5×10^9/L and the highest body temperatures 45±1.0 and 38±1.1°C, respectively.

Acute otitis media patient characteristics:

The study group of otitis media subjects (up to 5 years) were distributed into two groups, Group I,

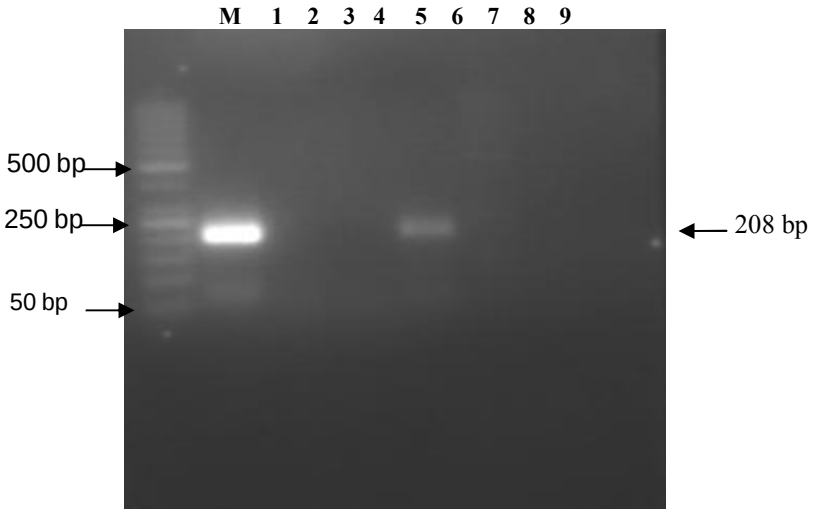

Fig. 1: Amplification of 208-bp fragment by PCR of DNA extracted from peripheral blood of pneumonia pediatric patients (Group A). Lane M, molecular size marker; lane 1 and 5 PCR positive samples and lane 2, 3, 4, 6, 7, 8, 9 PCR negative *S. pneumoniae* patients' blood samples

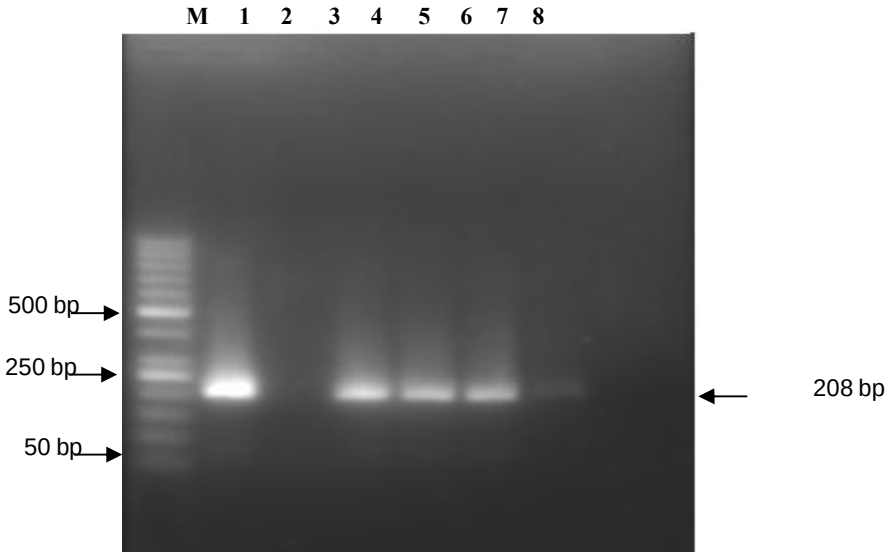

Fig. 2: PCR amplification results of Group B. lane M, DNA size marker; lane 1, 3-6 are positive *S. pneumoniae* patient's blood samples.

MEF pneumococcal culture negative (90 samples) and Group II culture positive (60 samples).

Specificity and sensitivity of PCR:

Among pneumonia patients of Group A, PCR gave a clear band of the expected molecular size in the nested-PCR (Fig. 1). The specificity of the PCR assay was demonstrated by its negative results for normal healthy persons. The sensitivity of the assay was evaluated with about 0.06 pg of purified S. pneumoniae genomic DNA. Amongst 44 pneumonia patient samples, 86.4% showed PCR positive results while 6.6% and 8.3% positive results were obtained in acute respiratory tract infection and pneumococcemia, respectively (Table 1). None of the samples of the controls was found to be positive by PCR and blood culture.

In Group B, nested-PCR protocol was found to be positive in 62 of the 80 pneumonia cases. Four (18%) patients with pneumococcemia and four (14.2%) with acute respiratory tract infection were positive for pneumolysin gene (Fig. 2, Table 1).

In other study, the results of PCR based amplification of the pneumolysin gene were compared with those obtained from culture method. Among 60 MEF culture positive samples (Group II), 52 showed the evidence of pneumolysin by nested-PCR. On the other hand, among 90 *S. pneumoniae* culture-negative MEF samples (Group I), pneumolysin gene amplification was observed in 8 samples (Table 2).

Discussion

S. pneumoniae is a common human pathogen that may be considered as a part of normal flora of the

Table 1: PCR results for peripheral blood samples from patients with clinical symptoms

Age group	Clinical presentation	No. of samples	No. (%) of pneumolysin negative samples by PCR	No. (%) of pneumolysin positive samples by PCR	*P*
Group A (1-6 months)	pneumonia	44	6(13.6)	38(86.4)	<0.05
	Pneumococcemia	24	22(91.7)	2(8.3)	<0.05
	ARTI	30	28(93.4)	2(6.6)	>0.05
Group B (above one year)	Pneumonia	80	18(22.5)	62(77.5)	<0.05
	Pneumococcemia	22	18(82)	4(18)	<0.05
	ARTI	28	24(85.8)	4(14.2)	>0.05

ARTI: Acute respiratory tract infection

upper respiratory tract, however, infants, elderly and deliberate patients with other compromising medical conditions of the respiratory tract, or decreased immunological infections are at great risk of acquiring pneumococcal disease. The spectrum of pneumococcal diseases changes in various age groups and different populations[4,11]. The diagnosis of pneumococcal infection requires *S. pneumoniae* isolation from infection sites by using insidious procedures such as lung puncture, pleural fluid aspiration, and middle ear fluid (MEF) aspiration; however, their presence in peripheral blood is an indirect confirmation. 20 to 30% of adults and 10% of children blood cultures are reported to be positive for pneumococcal pneumonia[12].

Pneumolysin is a species-specific protein toxin produced intracellularly by all clinically relevant pneumococcal strains. The expression of pneumolysin gene is anticipated to specify pneumococcal involvement in the disease, without considering the pneumococcal serotype. Therefore, pneumolysin is a good target for diagnosis since it is produced by all pneumococcal strains isolated from clinical samples[13] and its virulence properties are well known[14].

Molecular methods have been widely used for the diagnosis of several infectious diseases, including pneumococcal pneumonia, due to their high sensitivity, specificity and speed[15]. The purpose of this study was to develop a simplified molecular strategy for *S. pneumoniae* detection by DNA amplification in clinical specimens of children visiting different hospitals.

In Group A, among 44 pneumonia patient samples, 86.4% showed PCR positive results and only 2 positive results obtained in 30 acute respiratory tract infection samples. In Group B, 62 out of 80 pneumonia patients and 4 out of 28 acute respiratory tract infection patients were found to be pneumolysin positive (Table 1). Toikka et al[16] have described 44% pneumolysin PCR positive results out of 25 pneumococcal infected patients. Mayoral et al[17] reported that 10/24 serum samples of a group (children with definitive diagnosis of pneumococcal pneumonia confirmed by blood-culture) were positive when subjected to first round PCR. However, when the second round of nested-PCR was applied to the same samples, 20/24 (83.3%) were found to be positive. In our study, none of 60 healthy controls when analyzed by PCR, was found to be positive, indicating a good specificity and sensitivity of the PCR procedure.

Peripheral blood samples from PCR positive pneumonia patients (Group A and B) were collected prior and subsequent to antibiotic administration. Out of 100 patients under antibiotic treatment for less than 48 h, 3 were found to be PCR positive (Fig, 3). However, after antibiotic treatment for more than three days, pneumococcal DNA could not be detected. Even after 48 h antibiotic treatment, Dagan et al[18] did not detect pneumococcal DNA in the serum samples of the patients.

The diagnosis of acute otitis media (AOM) requires a sensitive and consistent method for deterrence of the disease. Bacterial culture and PCR-based assay was used to study the presence of *S. pneumoniae* in middle ear fluid samples from patients with AOM. Out of total 150 MEF culture samples, 60 cases were *S. pneumoniae* culture-positive and among them 52 samples exhibited the presence of pneumolysin gene by nested-PCR. On the other hand, out of 90 MEF culture-negative cases, 8 confirmed PCR positive results demonstrating the sensitivity and specificity of the molecular method to establish the involvement of pneumococcal pathogens in MEF samples of children with acute otitis media (Table 2). In other study, 47.1% PCR positive results for pneumolysin gene in MEF samples while 27.3% culture positive

Table 2: Comparison of S. pneumoniae culture and pneumolysin PCR results for MEF samples

S. pneumoniae culture	No. of Children	Pneumolysin PCR result No (%) Positive	Negative	P
Group I ([a]MEF-)	90	8 (8.8)	82 (91)	<0.001
Group II ([b]MEF+)	60	52 (86.6)	8 (13.3)	< 0.05

a: culture negative, b: culture positive; MEF: middle ear fluid; PCR: Polymerase chain reaction

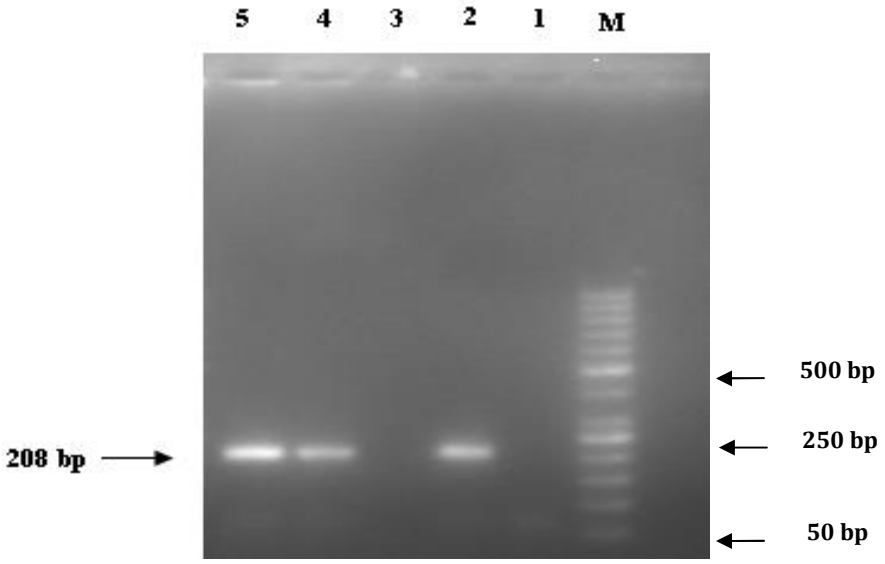

Fig. 3: Agarose gel electrophoresis of PCR-amplified products from antibiotic treated patient samples. Lane M, 50bp DNA size marker; lane 2, 4 and 5 are pneumolysin positive samples

results for the same samples are reported[19]. Virolainen et al[20] have described S. pneumoniae culture positivity in 33 (18%) and pneumolysin gene amplification in 51 out of 180 (28%) MEF clinical specimens. Only 2 out of 21 PCR-positives, S. pneumoniae culture-negative samples were positive for other otitis pathogens.

The statistical analysis results of observed data are presented in Table 1 and 2. Almost all studies demonstrated nearly ideal specificity and sensitivity estimates. Binomial test of proportionality performed on (SPSS 17) showed low *P* values (<0.05) that suggests pneumolysin gene to be a significant diagnostic genomic marker particular to *S. pneumoniae* using the PCR technique.

The diagnosis of pneumococcal contagion in children has been a challenge for years and largely relying on traditional culture techniques. In recent years to improve the analytic sensitivity and specificity of the diagnostic methods, numerous nucleic acid amplification technologies are developed for the rapid detection of widespread bacterial pathogens[21].

Polymerase chain reaction based assessment of pneumococcal pneumonia as compared to conventional methods, has made it a valuable tool in the etiologic diagnosis of pneumonia in the children visiting hospitals of Lahore. There are several factors that appreciate using the sophisticated, rapid and accurate diagnostic tools and specifically the methods designed for keeping economic and financial considerations of the developing country like Pakistan.

Conclusion

The present study describes a comparative analysis of sensitive nested-PCR and bacterial culture in pediatric patients with clinical and radiological indication of *S. pneumoniae* infection. Binomial test of proportionality demonstrate the sensitivity and reliability of PCR for rapid pneumonia evaluation.

Acknowledgment

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Conflict of Interest: None

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