Evaluation of polymerase chain reaction for direct detection of *streptococcus pneumoniae* in clinical samples & antimicrobial susceptibilities of the isolates

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ABSTRACT

Introduction: *Streptococcus pneumoniae (S.pneumoniae)* is an important cause of morbidity and mortality in people of extreme age. Drug resistant pneumococci are emerging and causing treatment failure. In the present study we evaluated Polymerase Chain Reaction (PCR) for rapid detection of *S. pneumoniae* directly from the clinical samples and antimicrobial susceptibilities of the culture isolates

Material & methods: A total of 90 clinical samples from sterile sites were cultured. Direct detection of *S.pneumoniae* from the samples was done using PCR. The isolates were tested for antimicrobial susceptibility by disc diffusion method and Minimum Inhibitory Concentration (MIC) of penicillin was determined by microbroth dilution method.

Results: Out of 90 samples 34 were positive for *S. pneumoniae* by PCR and 23 samples showed growth in culture. Of these 23 isolates, disc diffusion method showed that 61%, 0%, 13%, 35%, 83%, 4.3%, 0% of the isolates were resistant to penicillin, erythromycin, ciprofloxacin, tetracycline, co-trimoxazole, chloramphenicol, cefotaxime respectively and 13% erythromycin, 22% ciprofloxacin 9% tetracycline and 4.3% cotrimoxazole intermediate sensitive isolates were found. Only 4.3% isolates showed resistance to penicillin by microbroth dilution.

Conclusion: PCR is a sensitive & rapid method for detection of *S.pneumoniae*. Penicillin resistance in *S. pneumoniae* should be confirmed by MIC testing to avoid false reporting

Keywords: *Streptococcus pneumoniae*, Disc diffusion, PCR (polymerase Chain Reaction), MIC (Minimum Inhibitory Concentration)

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INTRODUCTION

Streptococcus pneumoniae (S.pneumoniae) is an important cause of morbidity and mortality among young children and elderly population. It is found to colonize the nasopharynx of healthy children and adults. Carriage rate among children is 40 % and among adults is 10 %. [1] Though it is a commensal in the upper respiratory tract but in immunocompromised patients & post viral infections, it can progress from the nasopharynx to the sterile sites such as lungs, blood and brain can lead to serious invasive pneumococcal disease (IPD). WHO has estimated that *S.pneumoniae* is responsible for 10-100 cases of IPD per 100,000 persons per year and more than a million deaths annually. [2] It is a common cause of otitis media; community acquired bacterial pneumonia, septicaemia and meningitis. It is also responsible for acute exacerbations of chronic obstructive pulmonary disease (COPD) and Asthma. [3] It is a fastidious organism. Isolation of *S.pneumoniae* from samples collected from sites in which only few organisms are present or in patients who have been given antibiotics is poor. Polymerase chain reaction (PCR) based method to detect *S.pneumoniae* from such clinical samples has been found to be useful. [4]

After the introduction of vaccines, prevalence of invasive pneumococcal infections has been reduced, but in developing countries mortality, especially in patients in whom diagnosis and antibacterial therapy is delayed, is still high. *S.pneumoniae* resistant to penicillin and other antibiotics have been reported from all over the world. However, there are very few reports of penicillin resistant isolates in India. Hence, continuous monitoring of drug resistance is required. The present study has been designed to isolate *S. pneumoniae* from various clinical samples and to determine their antibiotic susceptibility.

We planned to determine Minimum Inhibitory Concentration (MIC) for penicillin as significant difference in susceptibility by disc diffusion & MIC has been reported [5]. Direct detection of *S. pneumoniae* from sterile clinical samples by a PCR technique was also planned and to compare it with culture.

MATERIAL AND METHODS

This prospective study was conducted July 2006 to July 2008 at Vallabh Bhai Patel chest institute, Delhi, India

Clinical Specimens: Ninety clinical samples including pleural fluid , BAL, and tracheal aspirate aspirates from patients with pneumonia, respiratory illnesses like chronic obstructive lung diseases, bronchiectasis, and acute exacerbation of bronchial asthma, blood from cases of septicemia and pneumonia, cerebrospinal fluid (CSF) from cases of meningitis sent to the microbiology department of Vallabh Bhai Patel chest institute and VMMC & Safdurjung hospital for culture and identification of the pathogens were included in the study. Written informed consent was taken from all the patients. The study was ethically approved.

All the samples were inoculated on brain heart infusion broth (BHI broth), chocolate agar (CA), blood agar(BA), MacConkey's agar(MA) and incubated in 5% CO_2 at 37° C for 18 hours. Blood was collected in a ratio of I part blood to 5-10 parts BHI broth; inoculated for 24 hours and subcultured on CA, BA, MA and incubated in 5-10 % CO_2 at 37° C for 18 hours. The samples were stored at -20° C for DNA isolation and PCR test to be done later.

The alpha haemolytic colonies obtained on blood agar and chocolate agar were further identified by colony morphology, Gram staining, optochin susceptibility and bile solubility test. [6]

Antibiotic susceptibility testing: A total of 23 isolates obtained from culture were tested for antimicrobial susceptibility by Kirby-Bauer's disc diffusion test (Bailey & Scott, 2002). Penicillin, erythromycin, ciprofloxacin, tetracycline, co-trimoxazole, chloramphenicol and cefotaxime were used. The test organisms were grown by inoculating 2-3 colonies in nutrient broth and incubated at 37ºC overnight. The turbidity of the growth obtained was standardized to match the turbidity of 0.5 M Farland turbidity standards. Blood agar plate was inoculated using a swab impregnated with bacterial suspension.. Antibiotic impregnated discs (Hi Media) were placed on the surface of freshly inoculated plates The plates were incubated at 35- 37ºC in presence of 5-10% CO_2 for 18 hrs .The zone of inhibition was recorded and interpreted as sensitive, intermediate sensitive or resistant according to the Clinical and Laboratory Standards Institute (CLSI), guidelines.[7]

Minimum inhibitory concentration (MIC)
MIC of penicillin was determined by Micro broth dilution assay as per the method detailed in the CLSI guidelines. Briefly, the bacteria were grown on Muller-hilton agar with 5% sheep blood, overnight at 37° C with 5% CO_2. The bacterial colonies were suspended in 0.9% physiological saline to turbidity equal to 0.5 McFarland's standard (1.5 x 10^8 CFU/ml). This was diluted 1:30 before using. The In-house MIC panels containing

0.1 ml of lysed sheep blood-supplemented, cation adjusted Mueller-Hilton broth with different concentration of Penicillin were used. MIC panels were inoculated with 0. 1 ml of the bacterial suspension. and were incubated at 35ºC in 5% CO_2 overnight. The plates were read and the MIC recorded as the concentration of the first well showing no readily visible growth or haze as detected by the unaided eye. The isolates were labelled as sensitive, resistant or intermediate according to CLSI guidelines. *Streptococcus pneumoniae* ATCC 49619 and ATCC 31779 were used as control strains. [8]

Direct detection of *S. pneumoniae* by PCR:
Direct detection of *S. pneumoniae* from clinical samples was undertaken following the method of Matar et al. [9]

DNA Extraction: 100 µl of untreated sample or reference strains was overlaid with 2 drops of mineral oil in 0.5 ml micro centrifuge tube and boiled for 15 minutes.

PCR : PCR was initially done on DNA extracts using the universal primers i.e. RWO1, 5'-AAC TGG AGG AAG GTG GGG AT-3' and DG74, 5'-AGG AGG TGA TCC AAC CGC A-3', which amplify a 370-bp region of the 16S rRNA gene, highly conserved among all bacteria. The positive samples were further subjected to a PCR using the genus or species-specific primers of *S. pneumoniae* i.e. RDR 462 -5' AAC TGA GAC TGG CTT TAA GAG ATT A3'and RWO1. These primers flank a 132-bp region within the 16S rRNA gene of *S. pneumoniae*.(figure 1 & 2)

PCR Reaction: 100µl reaction volume→(10X PCR Buffer:10 µl, dNTPs:16 µl, Primer1 ;1 µl, Primer2 ;1 µl, Taq Polymerase(5 U/ml);0.5 µl, DNA Extract;10 µl, DDH2O;   61.5µl)
Conditions for PCR: Total Number of cycles-34, Per cycle (Denaturation 95ºC- 1 min, Annealing 55ºC- 1min, Extension 72ºC- 1min), Additional extension at 72ºC – 10 min.

RESULTS

Clinical specimens: The samples processed included CSF (26), blood (8), BAL fluid (43), pleural fluid (12) and tracheal aspirate (1). The age of patients ranged from 2 years to 95 years. Most of the CSF samples were from pediatric patients.

Isolates: All the alpha hemolytic, optochin sensitive and bile soluble isolates were identified as *S.pneumoniae.* A total of 23 *S.pneumoniae* isolates, (CSF-11, Pleural Fluid-3, Blood-8, Tr. Aspirate-1) were obtained from culture.

Antibiotic susceptibility testing: Out of 23 isolates, tested by disc diffusion test, high percentage of resistance was seen to co-trimoxazole 82.6%(19), oxacillin 60.8%(14) & tetracycline 34.7%(8), while low percentage resistance was seen to ciprofloxacin 13%(3), chloramphenicol 4.3%(1) and none showed complete resistance against cefotaxime & erythromycin. However, 21.7 % (5) ciprofloxacin, 13% (3)erythromycin, 8.6%(2)tetracycline & 4.3% (1) cotrimoxazole intermediate sensitive isolates were observed (table;1). MIC testing of Penicillin by microbroth dilution showed only 4 (17%) isolates to be intermediate sensitive while none of the isolate showed resistance to penicillin(table;2)

Direct detection of *S. pneumoniae* by PCR
Thirty four (37.7%) out of 90 clinical samples, were positive for *S. pneumoniae* by PCR. Out of these only 23(25.5%) were grown in culture. Eleven (12.2%) samples were positive by PCR but did not grow in culture (table;2). Considering culture as gold standard, PCR showed sensitivity & specificity 100% & 83.5% respectively.

DISCUSSION

Antibacterial resistance in *S.pneumoniae* is increasing worldwide, affecting principally beta-lactams and macrolides, prevalence ranging from 1% to 90% depending on the geographical area. A Global perspective of antimicrobial susceptibility shows an increased resistance to penicillin varying from 4.5% to 90.6%. [1, 10] Prior to 1995 all strains of *S. pneumoniae* isolated at a tertiary care hospital in South India were found susceptible to penicillin. However, since late 1995 strains of *S. pneumoniae* with intermediate resistance (1.3%) to penicillin have been observed. [11] After that various other Indian studies reported 4 to 20 % of resistance to penicillin [12, 13, 14]. Nonetheless, penicillin resistant pneumococci are less prevalent in India as compare to other Asian & western countries [10, 12]. There is a difference in resistance pattern of *S. pneumoniae* isolates observed in north & south Indian regions [5]. In the present study, 60% isolates showed resistance to penicillin by oxacillin disk diffusion test. Out of 9 isolates, which were resistant to penicillin by disc diffusion test, 8(34.7%) were found sensitive by MIC. Only 1 isolate from CSF sample showed resistance by micro broth dilution test. Hence, determining MIC of penicillin is a better indicator of true prevalence of penicillin resistance. [5, 15]

In the current study 13% isolates showed intermediate resistance, but no isolate was found resistant to erythromycin. Other studies from India, reported 0-4.2% of erythromycin resistance,

indicating, there is no need to introduce any other macrolide when pneumococcal etiology is confirmed[13,11,16]   A very high rate of erythromycin resistance was observed in other Asian countries, Vietnam (92.2%), Taiwan (86%), Korea (80.6%), Hong Kong (76.8%), and China (73.9%). [10] Resistance to chloramphenicol (4.3%) as observed in the present study correlated well with other studies. [11, 16, 17, 18] Resistance to tetracycline and co-trimoxazole(35% & 83% respectively) was also observed by other workers. [17, 14, 5]

Fluoroquinolone resistance has also started to emerge in countries with high consumption of antibiotics. In the present study 13 % of total isolates showed complete resistance while 22% were found intermediately sensitive to ciprofloxacin. Similar results were reported by other studies [5]. While, lower resistance (4%) was reported by Song JH *et al.* [10] In a study from U.S. 2.3% isolates had ciprofloxacin resistance. Very low resistance to cefotaxime & ceftriaxone has been reported by Goel R *et al.* [14] All the isolates were found sensitive to cefotaxime in our study.

As drug resistant pnemococcal infections are increasing, early and correct diagnosis of these infection is very important. Culture is gold standard and provides isolates for antibiotic susceptibility testing and serotyping, but it is less sensitive, time consuming and difficult to recover due to autolysis and prior antibiotic therapy. These lacunae of culture can be overcome by antigen based test and molecular tests, performed directly on the samples. Antigen based tests have lower sensitivity and specificity, may cross react with other streptococci and these tests can be positive for long time after treatment. [19] Molecular detection of *S.pneumoniae* has been achieved by PCR directly from various clinical samples. [4] Studies based on PCR have shown that autolysin gene (lytA) is more specific for *S. pneumoniae* than pneumolysin gene

(ply). [19] A major barrier to the direct use of body fluids in the PCR is the presence of inhibitory agents in the samples. [20] In the present study, a two-step 16S rDNA based PCR- assay has been used for the identification of *S. pneumoniae* in clinical samples. (figure1 & 2) *S. pneumoniae* was detected in 38% of the samples as against 25.5 % samples in culture proving that it is more sensitive than culture. Therefore 12.5 % cases that were missed by culture could be detected by PCR. Many other studies have demonstrated superiority of PCR using various gene targets (lytA, ply, capsular wzg/cpsA) over culture. [19]

Since the sensitivity of culture is low, taking it as a gold standard will obviously give a low specificity of the test in consideration, PCR in this case. PCR is rapid, results achievable in 5-6 hours, as compare to culture which takes 24-48 hours. It is specific because genus/species specific primers are used and false positives are unlikely. Another study confirmed the usefulness of 16S rDNA PCR in pleural fluids for diagnosis of empyema by identification of a large number of bacterial species. [21] Real time PCR allows monitoring of in-vitro amplification of DNA, eliminating nonspecific amplification and the need for gel electrophoresis, but, this is an expensive method and its routine use is restricted. [22]

Conclusion: PCR can be adopted in laboratories for rapid detection of *S.pneumoniae* from clinical samples. The penicillin and multi drug resistance to *S. pneumoniae* is increasing worldwide. Therefore judicious use of antibiotics & continuous surveillance for antibiotic resistance should be done to prevent the constant rise in drug resistant Pneumococci. The disc diffusion test should not be solely depended on while reporting penicillin sensitivity, as it could be misleading. MIC determination is a more sensitive test and must be performed before reporting penicillin resistance in *S.pneumoniae.*

Table 1: **Antibiotic susceptibility patterns of *s. pneumoniae* by disc diffusion test**

Disk Diffusion Test (n = 23)			
Antibiotics	**No. Of isolates (%)**		
	Sensitive	**Intermediate Sensitive**	**Resistant**
Oxacillin	9 (39)	0(0)	14 (60.8)
Erythromycin	20 (86.9)	03 (13)	0(0)
Ciprofloxacin	15 (65.2)	5 (21.7)	03 (13)
Tetracycline	13 (56.5)	02 (8.6)	8 (34.7)
Co-trimoxazole	03(13)	01 (4.3)	19 (82.6)
Chloramphenicol	22(95.6)	0(0)	1(4.3)
Cefotaxime	23 (100)	0(0)	0(0)

Table 2: MIC (MINIMUM INHIBITORY CONCENTRATION) OF *S. PNEUMONIAE* ISOLATES

Minimum inhibitory concentration, (n=23)			
Antibiotics (µg/ml)	**No. Of Isolates (%)**		
	Sensitive	**Intermediate Sensitive**	**Resistant**
Penicillin (Parenteral) Non-meningitis S ≤ 2, IS = 4, R ≥ 8 Meningitis S ≤ 0.06,IS=0.06-0.12, R ≥ 0.12	19(83)	4(17)	0(0)

S = sensitive, IS = intermediate sensitive, R = resistant

Table 3: CORRELATION BETWEEN RESULTS OF PCR AND CONVENTIONAL CULTURE FOR *S. PNEUMONIAE.* (N=90)

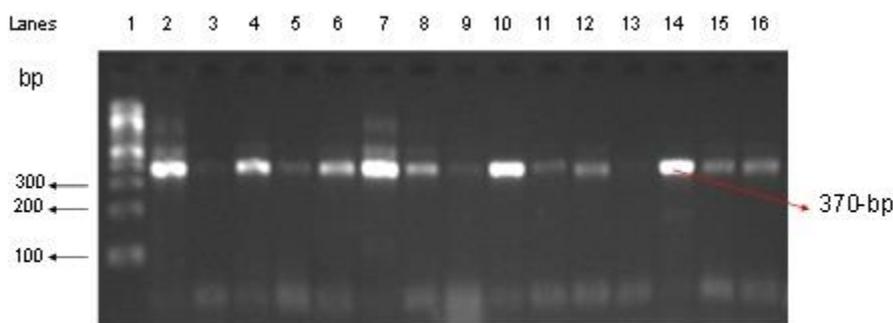| Samples	Culture positive	Culture negative	Total
PCR positive	23(25.5%) (Blood=8,CSF=11, PF=3, TA=1)	11(12.2%) (CSF=6, BAL=5)	34(37.7%)
PCR negative	0(0%)	56(62.2%) (BAL=38,P.F=9., CSF=9)	56(62.2%)
Total	**23(25.5%)**	**67(74.4%)**	**90**

Figure 1. Agarose gel of representative amplicons using:
Universal primers RW01 and DG74: lane1;100-bp ladder (Marker), lane3&13; PCR negative samples,lane2 (*S.pneumoniae* ATCC 49619) lane4 (*S. aureus*); positive controls for PCR , lanes,5,6,7,8,9,10,11,12,14,15&16; PCR-positive sample (370-bp band).
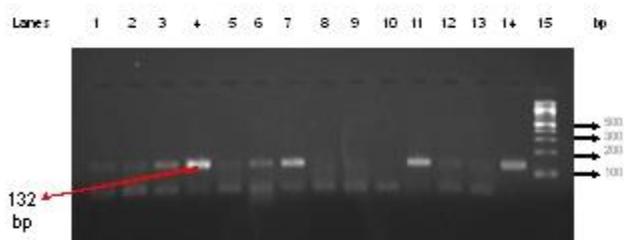
Figure 2. Agarose gel of representative amplicons using:
Genus/species-specific primers and a Universal primer; lanes 1,2,3,4,5,6,7,11,12;PCR-positive *S . pneumoniae* samples (132 bp), lanes 8,9,10,13;PCR negative samples, lane 14; positive control for PCR *(S. pneumoniae* ATCC 49619), lane 15;100-bp ladder (Marker).

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