Original Article

Characterization of *Streptococcus pneumoniae* isolates from India with special reference to their sequence types

Malini Shariff[1], Jyoti Choudhary[2], Shazia Zahoor[1], Monorama Deb[3]

[1]*Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India*
[2]*Ram Monohar Lohia Hospital, New Delhi, India*
[3]*Department of Microbiology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India*

Abstract

Introduction: *Streptococcus pneumoniae* is a major cause of mortality and morbidity in young children and the elderly. In the present study we evaluated antimicrobial susceptibilities, serotypes, and sequence types of pneumococcal isolates recovered in New Delhi, India.

Methodology: A total of 126 clinical isolates of *Streptococcus pneumoniae* were investigated. They were subjected to disk diffusion susceptibility testing, broth microdilution testing, serotyping and multilocus sequence typing.

Results: Broth microdilution assay showed that 5%, 20% and 23% of the isolates exhibited resistance to penicillin, erythromycin and ciprofloxacin, respectively. Serotypes19, 1 and 6 were more frequently isolated. Thirty per cent of the strains were comprised of serotypes 1, 3, 5, 19A and 7F, which are not included in the seven-valent vaccine. Fifty-nine isolates were typed using multilocus sequence typing. Thirty new sequence types were encountered in this study. Only one clonal complex with 4 isolates was seen; 11 clonal complexes and 96 sequence types (STs) were observed among 115 Indian isolates. Only 18 of the 96 STs were found globally, of which only 4 STs were found in many countries with larger numbers.

Conclusions: This study identifies the non-vaccine serotypes of *Streptococcus pneumonia*e circulating in India. It is important that an appropriate vaccine which covers all serotypes is used in the region.

Key words: *Streptococcus pneumoniae*; serotypes; sequence types; India

J Infect Dev Ctries 2013; 7(2):101-109.

(Received 23 January 2012 – Accepted 09 March 2012)

Introduction

Streptococcus pneumoniae is a commensal flora of the nasopharynx of humans. However, when it invades the bloodstream, it has the ability to cause serious infections ranging from otitis media, pneumonia, septicemia and meningitis, especially in children and elderly individuals. There are more than 90 serotypes identified. Some of the serotypes are known to cause more virulent infection than others. While various vaccines are available to prevent these infections, the 23-valent polysaccharide vaccine is commonly used, but due to poor immunogenicity in children, other multivalent conjugate vaccines are being developed and in use in the United States of America (USA), Canada, and European countries. A 7-valent conjugate vaccine (PCV-7) was introduced in the USA in 2000 and is widely used. Except for Korea and Taiwan, the PCV7 vaccine is not implemented in many Asian countries including India. Even if licensed in some of these countries, the coverage of the vaccine is very low [1]. PCV10 and PCV13 are also in use in some countries. To implement a vaccine effectively, one must be aware of the serotypes prevalent in that region so that an appropriate vaccine can be chosen. There are limited studies to indicate the serotypes of Indian strains. Molecular typing, especially of the sequence types, is available for only a very few strains [2,3]. The present study was undertaken to delineate the serotype, antimicrobial susceptibility pattern, and genotype of *S. pneumoniae* isolates from India and compare them with the global strains.

Methodology

Isolation and identification

This study was conducted between 2007 and 2010. Various clinical samples (cerebrospinal fluid, pleural fluid, blood, tracheal aspirate, nasopharyngeal samples, and sputum) obtained from patients suffering from meningitis, community acquired pneumonia, septicemia, or acute exacerbation of chronic obstructive pulmonary disease were processed and the *Streptococcus pneumoniae* isolates identified by

standard microbiological methods [4]. Briefly, these were cultured on 5% sheep blood agar plates and incubated overnight at 37°C with 5% CO_2. Draughtsman colonies with alpha hemolysis suspected to be *S. pneumoniae* were further confirmed by standard procedures for optochin susceptibility and bile solubility [4]. For sputum, only those isolates recovered in significant quantities (ascertained by quantitation of sputum) were considered as pathogenic [5]. Others were taken to be commensal.

Antibiotic susceptibility of the isolates

Isolates were tested for antimicrobial susceptibility by Kirby-Bauer's disk diffusion test. The test organisms were grown by inoculating two to three colonies in nutrient broth and incubating them at 37°C overnight. The turbidity of the growth obtained was standardized to match the turbidity of 0.5 McFarland's turbidity standards. A blood agar plate was inoculated using a swab impregnated with the bacterial suspension. Antibiotic impregnated disks (Difco, Franklin Lakes, NJ, USA) were placed on the surface of freshly inoculated plates. The plates were incubated at 35°C at 5% to 10% CO_2 for 18 hours. The zone of inhibition was recorded and interpreted per Clinical Laboratory Standards Institute (CLSI) guidelines [6].

Minimum inhibitory concentrations of the antimicrobials

Minimum inhibitory concentrations (MICs) of penicillin, erythromycin and ciprofloxacin were determined using the micro broth dilution assay per CLSI guidelines [7]. Briefly, bacteria were grown overnight on Muller-Hinton agar with 5% sheep blood, at 37°C with 5% CO_2. The bacterial colonies were suspended in 0.9% physiological saline to turbidity equal to the 0.5 McFarland's standard (1.5×10^8 CFU/ml). This was diluted 1:30 before use. The assay was performed in cation-adjusted Mueller-Hilton broth containing 0.1 ml of lysed sheep blood and various concentrations of antibiotics. 0.01 ml of the bacterial suspension was used to inoculate the MIC panels. These were incubated at 35°C with 5% CO_2 overnight. The microtitre plates were read and the antimicrobial endpoint recorded as 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 [7].

Serotyping of S. pneumoniae *isolates*

Serotyping of *S. pneumoniae* isolates was performed by latex agglutination test by coating the latex beads using the conventional antisera (Staten serum institute). It was performed by means of the "Chess Board Method" using the manufacturer's instructions. Briefly, the isolates were tested using the antisera present in pools A, B, C, D, E, F, G, P, Q, R, S, T and non-vaccine serotypes. Individual serotypes were determined by reactivity in two pools.

Multi Locus Sequence Typing (MLST)

Seven housekeeping genes (AroE, Gdh, Gki, RecP, Ddl, Spe and Xpt) were amplified using the method given by Gertz *et al*. and Enright *et al*. [8,9].

The amplified fragments were sequenced by Ocimum Biosolutions Ltd, Hyderabad, India. The assignment of alleles and sequence types was performed by the software available at the Multi Locus Sequence Typing website's pneumococcal page (http://www.mlst.net). Allelic combinations not already in the database were submitted and assigned new sequence types. The sequences were compared with the sequences of respective alleles downloaded from the website www.mlst.net. Clonal complexes (CC) were identified by grouping all isolates present in the *S. pneumoniae* database using the eBURST algorithm [10] with the software provided in the Multi Locus Sequence Typing website http://eburst.mlst.net). Clonal complexes consisted of sequence types that shared 6 of 7 alleles with at least one other sequence type in the complex and named after the putative founder (*i.e.*, the sequence type that had the greatest number of single-locus variants) of the group, or after the most frequent sequence type of the group. Sequence types that did not group with others in the database were defined as singletons.

Results

A total of 126 *S. pneumoniae* isolates, (18 CSF, 3 pleural fluid, 11 blood, 72 sputum, 3 tracheal aspirate, 2 throat swab, and 17 nasopharyngeal samples) were obtained from patients during the study period. The patients ranged in age from 2 to 77 years. Forty isolates were from children aged 2 to 12 years and the rest of the isolates were from adults. The majority of the pediatric isolates were from CSF and blood. Out of the 72 sputum isolates, 49 were recovered as the predominant pathogen while 23 isolates were commensal. In five cases other pathogens were isolated (three *Acinetobater*, one *Pseudomonas*, and one *Haemophilus*) in association with pneumococcus, which were also significant.

Antibiotic susceptibility of the isolates

Antibiotic resistance patterns are shown in Table 1. Among 126 isolates tested, a total of 41 isolates (33%) showed resistance to penicillin by oxacillin disk diffusion test. Ninety-eight (78%) isolates were sensitive, 8 (6%) isolates were intermediately sensitive, and 20 (16%) isolates were found resistant to Erythromycin. Furthermore, 103 (82%) and 38 (30%) isolates were resistant to co-trimoxazole and tetracycline respectively, while 17 (13%) isolates were found resistant to chloramphenicol. Nineteen (15%) isolates showed resistance while 25 (20%) isolates were found intermediately sensitive to ciprofloxacin, and 10 (8%) isolates were found resistant and 9 (7%) intermediately sensitive to cefotaxime. Forty-five (36%) isolates showed resistance to more than three drugs and were considered as multidrug resistant isolates. There was no difference in the preponderance of penicillin resistance seen in either the commensal or the pathogenic sputum isolates. Though *Acinetobacter* and *Pseudomonas* showed resistance to most groups of antibiotics, no resistance was observed to those groups in the pneumococcal isolates, which were isolated along with these.

Determination of minimum inhibitory concentration (MIC) of isolates found resistant to antimicrobials

MIC to penicillin and erythromycin was determined in 107 clinical isolates by micro broth dilution test. Five (5%) isolates, two each from CSF and nasopharyngeal samples and one from sputum, showed resistance to penicillin. Though 33% of the strains were resistant by disk diffusion test, only 5% showed resistance in this assay. Using the newer CLSI breakpoints most of the non-susceptible pneumococcal isolates were found to be sensitive; hence a marked reduction in the percentage of resistant isolates was found. Twenty-one (20%) isolates were resistant to erythromycin, which were resistant by disk diffusion test also. The MIC of ciprofloxacin was tested in 57 isolates, that were either resistant or intermediate-sensitive, and a few sensitive by disk diffusion test. Thirteen isolates (23%) showed resistance to ciprofloxacin and 20 (35%) were found intermediately sensitive; however, 5 isolates that were intermediately sensitive to ciprofloxacin by disk diffusion test showed low MIC levels and considered as susceptible (Table 2).

Serotyping of S. pneumoniae *strains*

Out of 108 *S. pneumoniae* isolates tested, the following serotypes were detected by the Chess Board Method: Serotypes 19 (26%), 6 (11%), 7 (10%), 1 (9%), 14 (7%), 9 (5%), 33 (4%), 17 (4%), 11 (2%), 3 (2%), 18 (1%), 23 (1%), 12 (1%), 32A (1%), 15B (1%), 22F (1%), 5 (1%), 29 (1%), non-vaccine type E (1%), F (1%) and H (7%). Eighty-two (80%) were of known vaccine types, out of which only 28 (34%) belonged to the 7-valent vaccine group, 44 (54%) belonged to PCV10, and 60 (73%) to PCV13, highlighting the fact that with the introduction of the 7-valent vaccine, serotypes other than this vaccine type are becoming prevalent. Twenty-six (24%) strains were untypable. The serotypes seen in this study mostly conform with those seen in an earlier study from India [11] except that serotype 19 is more prevalent than 1 and 6 in the present study.

Table 1. Antibiotic susceptibility patterns of *S. pneumoniae* isolates by disk diffusion test

Disk diffusion test (n = 126)			
Antibiotics	**Number of isolates (%)**		
	Sensitive	**Intermediate sensitive**	**Resistant**
Oxacillin	85 (67)	-	41 (33)
Erythromycin	98 (78)	8 (6)	20 (16)
Ciprofloxacin	82 (65)	25(20)	19 (15)
Tetracycline	81 (64)	06 (5)	38 (30)
Co-trimoxazole	21 (17)	02 (1.5)	103 (82)
Chloramphenicol	102(81)	7(6)	17(13)
Cefotaxime	107 (85)	9 (7)	10 (8)

Table 2. MIC (minimum inhibitory concentration) of *Streptococcus pneumoniae* isolates

Minimum inhibitory concentration			
Antibiotics	**Number of isolates (%) n = 107**		
(µg/ml)	**Sensitive**	**Intermediate sensitive**	**Resistant**
Penicillin (Parenteral) Non-meningitis S ≤ 2, IS = 4, R ≥ 8 Meningitis S ≤ 0.06, R ≥ 0.12	102(95)	0	5 (5)
Erythromycin S ≤ 0.25, IS = 0.5, R ≥ 1	86 (80)	0 (0)	21(20)
Ciprofloxacin * S ≤ 1, IS = 2, R ≥ 4	24 (42)	20 (35)	13 (23)

S = sensitive; IS = intermediate sensitive; R = resistant

*Total tested = 57 (All isolates showing resistance and Intermediate-sensitive and a few sensitive by disc diffusion test)

Multi Locus Sequence Typing

The sequence types and their comparisons with other STs in the MLST database are given in Table 3. Fifty-nine isolates were typed. Out of these 29 were of known sequence types while the remaining 30 showed new sequence types. Twenty-five out of these 30 were submitted to the MLST site and were assigned new ST numbers. The new types were variants of the existing sequence types differing in one or more loci. This observation shows that the serotypes are continuously evolving. ST types 303 (4 isolates), 4217 (2) 1811 (2) and 1669 (4), 6899 (2) and 6896 (2) each had more than one isolate. Some of the new STs in this study are single locus variants (SLVs) of an earlier new type found in India. For example, ST 1702, which was earlier found in the study, was a sensitive clone which has evolved into SLV ST6896 which is resistant to co-trimoxazole. All other types had a single isolate. There was only one clonal complex seen that was comprised of four isolates; the rest were singletons.

Discussion

This study characterizes the Indian isolates with respect to antimicrobial sensitivity, serotypes, and sequence types. Using the new breakpoints for non-meningeal isolates, many of the non-susceptible isolates were found to be sensitive to penicillin and hence only 5% of the isolates were resistant to penicillin, which is low in comparison to the resistance reported by several investigators from other countries [3,12]. Two out of the five resistant isolates were from CSF; however, it is interesting to note that two isolates from nasopharyngeal samples of healthy children were resistant to penicillin with a high MIC of > 4. These were also resistant to erythromycin. Dissemination of these resistant strains in the community can lead to treatment failure to penicillin. Multidrug resistance was seen in 36% of the isolates, which is comparable to resistance rates seen in studies from North America (9% to24%) and Europe (0 to 43%) [3,13,14]. However, this rate is much lower than the overall resistance rate of 59.3% reported more recently from Asian countries, with the highest seen in China (83.3%) followed by Vietnam (75.5%). In the present study, resistant isolates were not restricted to any particular serotype; these results differ from those seen in other studies which have reported more than 60% of 19A and 35B to be non-susceptible to penicillin [12,15-17]. In the present study, 20% of the isolates were resistant to erythromycin, and this rate is very low compared to the high resistance rate of 70% seen in other Asian countries [18].

It is evident from the serotyping data that only 34% of our strains would be covered by the 7-valent vaccine. Similar results were seen in investigations from other Asian countries, which reported 52.5% of the strains being covered [18]. An additional 20% each would be covered by the 10-valent and 13-valent conjugate vaccines. In spite of non-introduction of the vaccine in the regular immunization programme in India, non-PCV7 vaccine serotype 19A (data not shown), 7F and 1 were seen in 6%, 10% and 9% of the

Table 3. Clinical and molecular characteristics of *Streptococcus pneumoniae* isolates from India and their comparison with isolates recovered from other countries

S. No.	ST (Number)	Serotype	Antibiotic resistances[3]	Identical or related genotypes [SLV] from isolates recovered from other countries / associated serotypes / resistances / countries / clinical presentation / age
1	1669¶ (4)	6A	SXTR,TETR	Identical/6A,C/TETR/India, Australia / pneumonia, sinusitis
2	1671¶	32A	SXTR,CHLR TETR	Only 5 identical alleles in closest
3	1700¶	3	TETR,CHLR	ST458,ST2633 [SLV] /3,33F
4	1701¶	7C	PENI, SXTR, TETR	Identical /19A /TETR/Thailand/ carriage
5	1702¶	7F	None	STs 28,253,5067,6896 [SLVs]/8,18, 7F, NT/ Netherlands, India/
6	1725¶	15B	SXTR,TETR	ST1210 , 4209,6020,6038 [SLV] / 15B /SXTR /Kenya, India, Nepal-2/ carriage, blood, carriage /adult [HIV+]), child
7	1726¶	29	SXTR, TETR, CHLR	Only 4 identical alleles in closest match
8	1759¶	7F	SXTR,TETR, CHLR	Only 5 identical alleles in closest match
9	6891¶	7F	None	STs1167,3684 [SLVs]/19/ sensitive/Norway, Spain, Netherlands/ meningitis, carriage/
10	6892¶	7F	SXTR,TETR	Only 5 identical alleles in closest match
11	6893¶	14	SXTR, ERYR, TETR	ST 1545 [SLVs]/19F/ ERYR, TETR/Germany, Poland/meningitis, septicemia
12	6894¶	untypable	SXTR, ERYR TETR,CIPIS	Only 5 identical alleles in closest match
13	6895¶	17	SXTR,TETR	Only 4 identical alleles in closest match
14	6896¶ (2)	7F	SXTR	ST1702 [SLV]/7F/sensitive/India/
15	6897¶	untypable	None	ST5275,6720 [SLV]/ 4, 19F/Singapore, Ethiopia
16	6898¶	33	SXTR, TETR	Only 5 identical alleles in closest match
17	6899¶(2)	untypable	SXTR,TETR, CIPI	Only 4 identical alleles in closest match
18	6900¶	7F	SXTR	ST 5067 [SLV]/ NT, 7F/ SXTR/India
19	6901¶	21	SXTR	ST 5103 [SLV]/21/TETR/Thailand, Nigeria /carriage
20	6902¶	6	SXTR, CIPI	Only 5 identical alleles in closest match/Gambia/carriage
21	303 (4)	1(3),19F	TETR, CHLR, SXTR	Identical /1/sensitive/Ghana, Niger, Bangladesh, Germany/septicaemia, meningitis, pneumonia
22	1475	27	None	Identical/27/sensitive/Poland/Meningitis
23	5067	7F	SXTR	Identical/NT/sensitive/India, ST 28,253,1702[SLVs]/Netherlands, Germany, India
24	3804	11	SXTR, ERYR, CIPI	Identical /11/Nigeria, Togo/
25	5407	25F	TETR	Identical /25F/TETR,ERYR/India/bacteremia
26	4661	11A	SXTR,TETR, CIPI	Identical/11A/TETR, ERYR/China ST280[SLV]/TETR/Nigeria, Thailand, Czech Republic, Vietnam
27	6040	39	TETR	Identical/39/TETR/Nasopharynx/Nepal
28	5445	6	SXTR,TETR, CHLR	Identical/6B/TETR,CHLR/India

Table 3 Continued. Clinical and molecular characteristics of *Streptococcus pneumoniae* isolates from India and their comparison with isolates recovered from other countries

S. No.	ST (Number)	Serotype	Antibiotic resistances[3]	Identical or related genotypes [SLV] from isolates recovered from other countries / associated serotypes / resistances / countries / clinical presentation / age
29	4217 (2)	19A, 6	SXTR	Identical/6B/India
30	5392	20	SXTR	Identical/20/sensitive/Kenya/carriage
31	688	19F	SXTR	Identical/19F/sensitive/ UK
32	989	12	SXTR	Identical/12/Kenya, Ghana, Qatar, Niger, Gambia
33	1811 (2)	9 V	SXTR, TETR, CHLR , CTXR	Identical/9V/TETR,CHLR/USA/septicemia
34	4936	14	CIPI	Identical/6A/sensitive/Thailand/carriage
35	3517	19F	None	Identical/19F/Bangladesh/carriage
36	236#	19F	SXTR	Identical PMEN Clone/19F/TETR/Taiwan
37	1796	19A	ERYR	Identical/35B/TETR, CHLR/USA/Septicaemia
38	5192	14	TETR	Identical/14/India/pneumonia
39	3518	19F	PENR, ERYR, TETR, CHLR CTXR	Identical/19F/Bangladesh/Carriage
40	1670	3	CIPR	Only 3 identical alleles in closest match
41	289#	5	SXTR ,TETR	ST289 [identical] /5/ TETR, CHLR/ Colombia/ meningitis, pneumonia/ no ages given
42	63#	14	None	ST63 [identical]/15A,19A,19F,23F /PENI,ERYR, TETR / many countries./ 3 – 82 yrs
43	1294	22F	CHLR, CIPR, LFXR	ST1294 [identical]/22F/sensitive/ United States/bacteremia, carriage

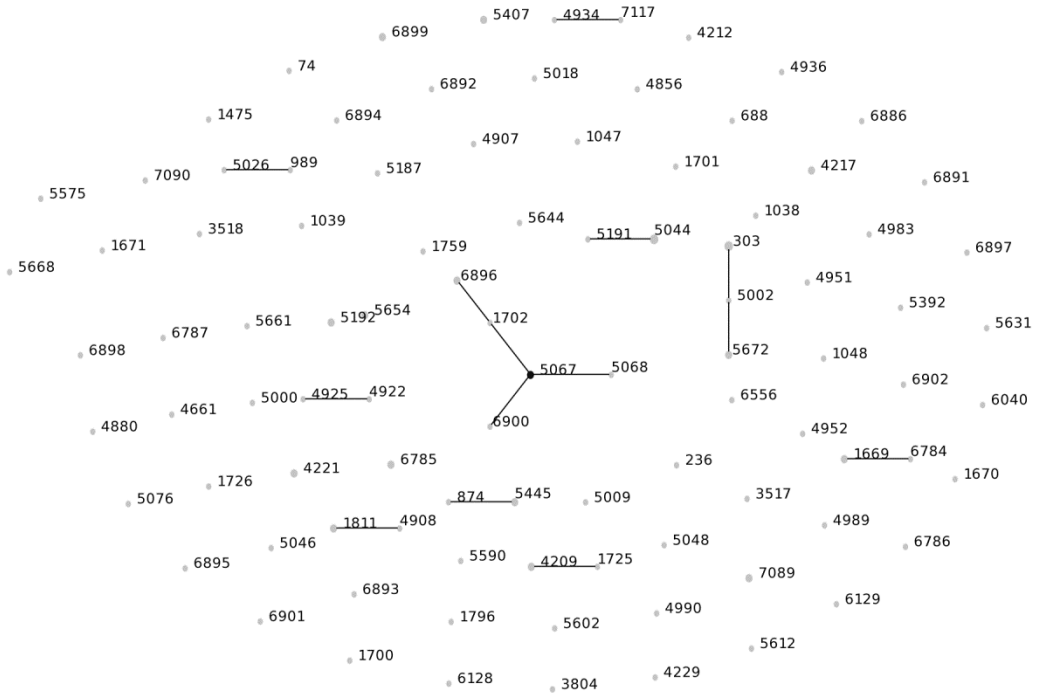
S. No. = Series number
¶New STs found during this study
# ST corresponding to 3 of 26 antibiotic resistant isolates recognized by the Pneumococcal Molecular Epidemiology Network
CIP = ciprofloxacin; LFX = levofloxacin; CHL = chloramphenicol; PEN = penicillin; SXT = trimethoprim sulfamethoxazole; TET = tetracycline; CTX = cefotaxime
I = intermediate resistance; R = resistant; SLV = single locus variant

strains respectively. The results are concordant with those seen in other Asian countries [18]. Hence the 13-valent pneumococcal vaccine would be a better choice.

The MLST database has a total of 115 Indian isolates (including ours) with 96 STs (as on 25 October 2011, 17:16:13 IST). Among these, only 10clonal complexes (CC) comprised of 38 isolates were seen; the rest all were singletons (Figure 1). These data show the diversity even among the Indian isolates. CC5067 (Group 1) was the largest with 5 STs having 8 isolates. Four of the 5 STs were isolated in this study. This CC is included in the Group 52 of the global strains (Figure 2). The predictor founder of this group (5067) is from India and is a double locus variant (DLV) of 27, which was an older ST recognized in 1995 from Denmark and an SLV of 28, which was recognized in 1994 from the Netherlands and later in 2005 from Germany. Four SLVs of this sequence type have originated in India since, suggesting that though initially acquired from Europe, the ST is evolving and giving rise to newer STs which are restricted to India.

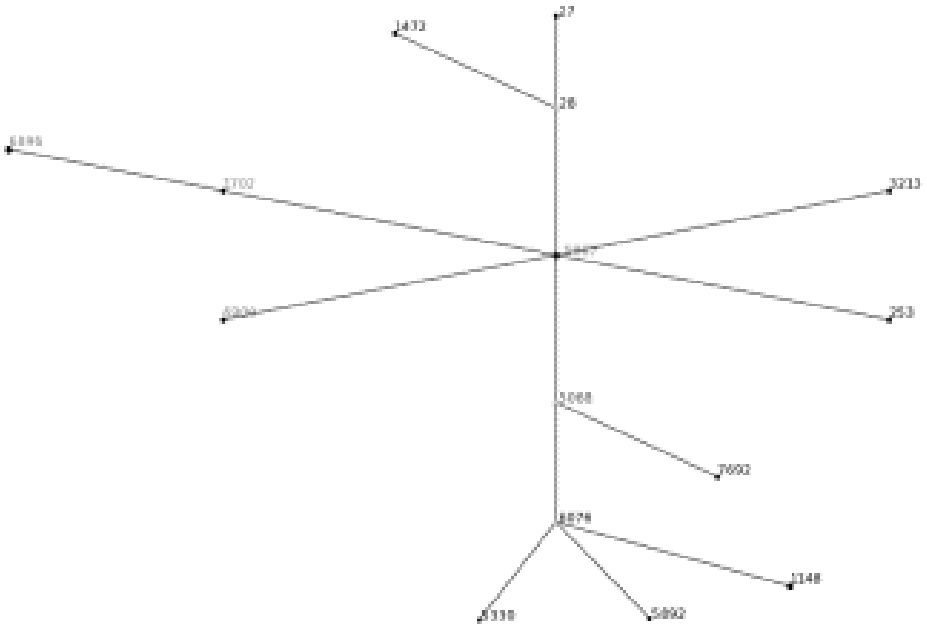
Comparing the Indian STs with the global scenario, it was observed that only 18 of the 96 STs from India were found globally. Among these only 4 STs (303, 989, 236 and 63) were predominantly found in larger numbers in many countries. All other STs were restricted to a few countries including India. Most of these were from Thailand, African countries such as Nigeria, Kenya and Ghana, and neighbouring countries of India including Nepal, Bangladesh and China (www.mlst.net). Most of the STs seen in African countries are also seen in India as such or as their SLVs, and this correlation may be explained by the travel between India and these countries and hence the dissemination of the African strains.

Figure 1. Population snapshot of115 Indian isolates of *Streptococcus pneumoniae* based on eBURST analysis



Ten clonal complexes seen among the 115 Indian *Streptococcus pneumoniae* isolates. CC 5067 (Group I) is the largest with 5 isolates.

Figure 2. Population snapshot of Group 52 (Predictor founder 5067) of Global isolates (inclusive of Group 1 of Indian isolates**)** of *Streptococcus pneumoniae* on eBURST analysis



CC 5067 comprised of 14STs, including 5 STS (5067, 5068, 6900, 1702 and 6895) from India

Isolates showing the same serotype but different sequence types and vice versa, seen across the globe, were observed in this study, also showing that they are genetically different. For example, isolates with serotype 7F showed 6 different sequence types in this study. Similarly, ST 303 and ST 4217 were present in multiple serotypes.

Out of the three STs, 63, 289 and 236 belonging to the PMEN clone, resistance pattern and serotype of new Delhi, were concordant with corresponding features of PMEN clones ST289 (Colombia ^{5}-19) and ST 236 ((Taiwan^{19F}-14). However, ST 63 isolate in this study was antibiotic sensitive and serotype 14, while the corresponding PMEN clone (Sweden15A-25) was resistant and of serotype 15A. ST 63 is seen to be present in various serotypes including 15A, 14, 19A 23F and 19F. The presence of this ST in non-vaccine serotypes, especially 19A, can eventually replace the vaccine serotypes and have the potential to cause disease, as seen in the present study also (unpublished data). After the introduction of pneumococcal vaccine, certain non-vaccine serotypes (11, 15, 33, 35, and 38 *etc*.), which were normal commensals of the naso-pharynx, have been shown to cause serious invasive infections [19,20]. Hence continuous monitoring of the isolates worldwide is required to implement appropriate vaccines in a particular geographical area.

Conclusion

The majority of the isolates obtained in this study were sensitive to penicillin and belonged to non-7-valent vaccine serotypes. Most of them were of unique sequence types, showing that they were very diverse. Therefore, these facts have to be kept in mind before treating patients and also before introducing the pneumococcal vaccine in India.

Acknowledgements

The funding was provided by Department of Biotechnology, Govt. of India vide:BT/PR9412/MED/29/26/2007 Dtd. June 30th 2008.

References

1. Shin J, Boek JY, Kim H, Song JH. Ko KS (2011) Predominance of DT320 among St*reptococcus pneumoniae* serotype 19A isolates from 10 Asian countries. J Antimicrob Chemother doi:10.1093/jac/dkr048.
2. Shariff M, Thukral SS, Beall B (2007) New multilocus sequence types of *Streptococcus pneumoniae* isolates from patients with respiratory infections in India. Indian J Med Res 126: 161-164.
3. Song JH, Jung SI, Ko KS, Kim NY, Son JS, Chang HH, Ki HK, Oh WS, Suh JY, Peck KR, Lee NY, Yang Y, Lu Q, Chomgthaleong A, Chiu CH, Lalitha MK, Perera J, Yee TT, Kumarasinghe G, Jamal F, Kamarulzaman A, Parasakthi N, Van PH, Carlos C, SO T, Ng TK &Shibl A (2004) A high prevalence of antimicrobial resistance among clinical *Streptococcus pneumoniae* isolates in Asia (an ANSORP Study). Antimicrob Agents Chemother 48: 2101-2107.
4. Overview of Bacterial Identification Methods & Strategies. In Forbes BA, Sham DF, Weissfeld AS (Eds): Bailey & Scott's Diagnostic Microbiology, 11th edn, Andrew Allen. 261-283.
5. Monroe PW, Muchmore HG, Felton FG, Pirtle JK (1969) Quantitation of microorganisms in sputum. Applied Microbiol 18: 214-220.
6. National Committee for Clinical Laboratory Standards (2004) Performance standards for antimicrobial susceptibility testing, fourteenth informational supplement. M100-S14.NCCLS, Wayne, PA.
7. Clinical and Laboratory Standards Institute. Performance standards for Antimicrobial susceptibility testing; eighteenth informational supplement M100-S18, Wayne, PA: 2008.
8. Gertz RE, McEllistrem MC, Boxrud DJ, Li Z, Sakota V, Thompson TA, Facklam RR, Besser JM, Harrison LH, Whitney CG, Beall B (2003) Clonal distribution of invasive pneumococcal isolates from children and selected adults in the United States prior to 7-valent conjugate Vaccine introduction. JClinMicrobiol 41: 4194-4216.
9. Enright MC and Spratt BG (1998) Amultilocus sequence-typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 144: 3049-3060.
10. Feil EJ, Li BC, Aanensen DM, Hanage WP. Spratt BG (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 186: 1518-1530.
11. Invasive Bacterial Infection Surveillance (IBIS) Group and International Clinical Epidemiology Network (INCLEN) (1999) Prospective multicentric hospital surveillance of *Streptococcus pneumoniae* disease in India. Lancet 353: 1216-1221.
12. Beall B, McEllistrem MC, Gertz RE Jr, Boxrud DJ, Besser JM, Harrison LH, Jorgenson JH. Whitney CG (2002) Emergence of a novel penicillin-nonsusceptible, invasive serotype 35B clone of *Streptococcus pneumoniae* within the United States. J Infect Dis 186:118-122.
13. Lynch JP, 3rd and Zhanel GG (2009) *Streptococcus pneumoniae*: does antimicrobial resistance matter? Semin Respir Crit Care Med 30: 210-238.
14. Lynch JP, 3rd and Zhanel GG (2010). *Streptococcus pneumoniae*: epidemiology and 461 risk factors, evolution of antimicrobial resistance, and impact of vaccines. Curr Opin 462 Pulm Med 16: 217-225.
15. Hanage WP, Huang SS, Lipstitch M, Cynthia JB, Godoy D, Pelton SI, Goldstein R, Huot H, Finkelstein JA (2007) Diversity and antibiotic resistance among non-vaccine serotypes of *Streptococcus pneumonia*e carriage isolates in the post-heptavalent conjugate vaccine era. J Infect Dis 195: 347-52.
16. Jacobs MR, Good CE, Sellner T, Bajaksouzian S, Windau A, Anon JB (2007) Nasopharyngeal carriage of respiratory pathogens in children undergoing pressure equalization tube placement in the era of pneumococcal protein conjugate vaccine use. Laryngoscope 117: 295-298.

17. Moore MR, Gertz RE Jr, Woodbury RL, Genevieve A, Gallagher B, Schaffner W, Lexau C, Gershman K, Reingold A, Fartley M, Harrison LH, Hadler JL, Bennet NM, Thomas AR, McGee L, Pilishvili T, Brueggemann AB, Whitney CG, Jorgensen JH, Beall B (2008) Population snapshot of emergent *Streptococcus pneumoniae* serotype 19A in the United States (2005) J Infect Dis 197: 1016-1027.
18. Kim AH, Song JH, Chung DR, Thamlikitkul V, Yang Y, Wang H, Lu M, So TM, Hsueh PR, Ysin RM, Carlos CC, Pham HV, Lalitha MK, Shimono N, Perera J, Shibl AM, Baek JY, Kang CI, KO KS, Peck KR (2012) Changing trend of antimicrobial resistance and serotypes in *Streptococcus pneumoniae* in Asian countries: an ANASORP study. Antimicrob Agents Chemother doi:10.1128/AAC.05658-11.
19. Kaplan SL, Mason EO Jr, Wald ER, Schutze GE, Bradley JS, Tan TQ, Hoffman JA, Givner LB, Yogev R, Barson WJ (2004) Decrease of invasive pneumococcal infections in children among 8 children's hospitals in the United States after the introduction of the 7-valent pneumococcal conjugate vaccine. Pediatrics 113: 443-449.
20. Perez A, Gimenez M, Sala P, Sierra M, Esteve A, Rodrigo C (2011) Increase in invasive non-vaccine pneumococcal serotypes at two hospitals in Barcelona: was replacement disease to blame? ActaPediatrica 100: 1572-1575.

Corresponding author
Dr. Malini Shariff
Associate Professor
Department of Microbiology
Vallabhbhai Patel Chest Institute
University of Delhi
Delhi -110 007
Telephone: +91-11-27402424 Fax: +91-11-27667420
Email: malini.shariff@gmail.com

Conflict of interests: No conflict of interests is declared.