

Characterization of invasive Pneumococcal isolates recovered from patients in two Selected Hospitals, Ethiopia

Surafel Fentaw, Amete Mihret, Rajiha Abubeker, Negga Asamene, Teklil Biza, Degefu Beyene, Elias Seyum, Abebe Aseffa, Yonas Mekonen, Tesfa Addis, Eyasu Tigabu and Sintayehu Abate Temesgen

Ethiopian Public Health Institute

Corresponding author: sura4f@gmail.com, P.O.Box:- 1242, Tel: +251-911 181729, Addis Ababa, Ethiopia

Abstract

Back ground: Pneumococcus is a major public health challenge with high frequency of infection and serotype diversity.

Objective: This study aims at serotyping, sequence typing and antimicrobial susceptibility profiling of pneumococcal isolates circulating in selected health facilities of Ethiopia.

Methods: A total of 228 cerebrospinal fluid samples were collected from hospitalized patients at University of Gondar Hospital and Black Lion Specialized Hospital during June –December, 2012. Samples were processed using both conventional and modern microbiological techniques. Serotype was determined by Quellung reaction. Antimicrobial susceptibility test was performed using E-test. Real Time –polymerase chain reaction and Multilocus sequence typing was also done for confirmation test and genotyping of isolates, respectively.

Results: Sixty eight pneumococcal isolates were recovered (26.3%) from the total 228 processed samples. Of these, 15 serotypes were identified, with serotype 1 (21%) being the leading followed by 19F and 20 (each 10%), and serotype 14 (8%). More than one serotype was identified in seven of the samples. Seventeen sequence types (mainly, ST1203, ST1475, ST217, and ST6451) were identified, of which seven of them were novel. RT-PCR analysis of 140 culture negative cerebrospinal fluid samples detected 12 *Neisseria meningitidis*, nine *Streptococcus pneumoniae* and 2 mixed strains.

Conclusion: Serotype 1 was predominantly found in suspected meningitis patients admitted in the study hospitals. Novel variants of pneumococcal sequence types that are new for the *Streptococcus pneumoniae* MLST database were also identified.

Key words: Pneumococcus, *Streptococcus pneumoniae*, serotype, MLST, sequence type

Introduction

The bacterium *Streptococcus pneumoniae* (pneumococcus) is the main cause for different types of diseases like otitis media, lower respiratory tract infection, bacteremia and meningitis all over the world including developing countries like Ethiopia (Ortqvist et al. 2005). Invasive pneumococcal diseases cause more than 800,000 deaths worldwide per year (O'Brien et.al. 2005; WHO 2003). Currently, based on the immunochemistry of polysaccharide capsule, there are around 94 different capsular types that have been identified and out of these, 15 to 20 types of serotypes are pathogenic while the remaining can live commensally. The distribution of the serotypes differs based on geographical location, season, immune status of the individual and age group (Todar et al. 2010). The increment of antimicrobial resistant pneumococcal strains is one of the public health concerns worldwide (Martin et al. 2003). As well as for the introduction of vaccine for intervention program, there should be evidence based data which can determine the characteristics of pneumococcal isolates based on their capsular immunogenicity serotyping which circulate in the community.

The conjugated polysaccharide capsule should contain at least sixty to eighty percent of serotypes in the community (Adegobla et al. 2006). Knowledge of serotype distribution, antimicrobial susceptibility patterns and clonality of circulating strains is important in the selection and implementation of vaccine (Mudhune 2009; WHO 2003). Not only information on the serotyping and antimicrobial susceptibility testing of pneumococcus in Ethiopia is limited, but data on clonal relationship among the different serotypes is also very scarce. Therefore, this study was aimed to define the serotypes, genotype and antimicrobial susceptibility profile of *S. pneumoniae* isolated from patients in selected health facilities of Ethiopia.

Materials and methods

Study population and samples collection: Samples were collected from two hospitals, namely, Black Lion Specialized Hospital (Addis Ababa) and Gondar University Hospital (Gondar). Both institutions are public facilities that serve an estimated population of 8.15 million according to the central statistics agency report (CSA, 2000).

Routine Cerebrospinal fluids (CSF) diagnostic samples for bacterial culture were collected between June and December 2012 from admitted patients and then transported to the Ethiopian Public Health Institute (EPHI) using di-phasic Trans Isolate media at ambient temperature and cold temperature in cryovial tubes for Polymerase Chain Reaction (PCR) analysis.

Bacterial culture, species identification and serotyping: All CSF specimens were analyzed by culture for the presence of *Streptococcus pneumoniae* immediately upon arrival at the EPHI. The specimens were inoculated on blood agar (Oxoid, Bangshare, England) containing 5% sheep blood, and Chocolate agar plates (Oxoid, Bangshare, England). The plates were incubated at 37°C under 5-8% CO₂. Isolates were confirmed as *S. pneumoniae* by Gram stain followed by optochin (Oxoid, Bangshare, England) and/or bile solubility tests (Difco) (WHO). Isolated pneumococcal species were serotyped at the Norwegian Institute of Public Health in Oslo. Serotyping was done using capsular and factor typing antisera observing capsular swelling microscopically (SSI 2001).

Antimicrobial susceptibility testing: For all confirmed *S. pneumoniae* isolates, antimicrobial susceptibility was performed by determining minimum inhibitory concentration (MIC) using E-test (Biomeriux, France).

The European committee on antimicrobial susceptibility testing (EUCAST) guideline was used for interpretation.

Genotyping: Multilocus Sequence Typing (MLST) was performed using internal fragment of the seven housekeeping genes (aroE, gdh, gki, recP, spi, xpt, and ddl). These genes were sequenced by using an automated protocol with Hitachi and Mega BACE DNA sequencer (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). Alleles were assigned by comparing nucleotide sequence data at a particular locus to all known alleles at that locus (Mitchel et.al. 2003). Sequence types (STs) were assigned combining the seven alleles with reference to the *S. pneumoniae* MLST database (www.mlst.net).

Real-time PCR: Real-time PCR analysis was performed for all culture negative CSFs. Bacterial DNA was extracted from 200 µl of each CSF sample using QIAamp® DNA mini kit. Real-time PCR for detection of pneumococcus, Hib and meningococcus was performed using positive control of targeted organism and negative control (nuclease free water) was run in parallel by ABI Fast Real-Time PCR machine. List of primers and probes those used in real time PCR analysis are indicated in table 1.

Table 1: Real Time PCR primers and probes used for culture negative CSF

Target	Primer or Probe Name	Real-time Primers and Probes Nucleotide Sequence (5' to 3')	Probe modification
<i>N. meningitides</i> (ctrA)	ctrA-F ctrA-R ctrA-P	GCTGCGGTAGGTGGTTCAA TTGTGCGGATTTGCAACTA CATTGCCACGTGTCAGCTGCACAT	5'-FAM, BHQ1-'3
<i>H. influenza</i> (hpd)	hpdF822 hpdR952 Pb896i	GGTTAAATATGCCGATGGTGTG TGCATCTTTACGCACGGTGTA TTGTGTACACTCCGTXGGTAAAGAACTGCAC	5' FAM, BHQ1-dT on X, 3' SpC3
<i>S. pneumoniae</i> (lytA)	F373 R424 Pb400i Thermal cycling condition	ACGCAATCTAGCAGATGAAGCA TCGTGCGTTTTAATTCCAGCT TGCCGAAAACGCXTGATACAGGGAG 95°C for 20 Sec 95°C for 3 se 60°C for 30 sec	5' FAM, BHQ1-dT on X, 3' SpC3 1cycle 45 cycles

Statistical Analysis: Descriptive statistical analysis, cross tab and association between dependent and independent variables were done using SPSS software version 20. Statistical significance level was set to be P <0.05. Ethical clearance was obtained from the Norwegian Ethical Review Board (REK) and Scientific and Ethical Review Office of EPHI.

Results

Specimens: CSF samples from a total of 228 patients were processed in the study. Out of these, 61 (13.3%)

were positive for *S. pneumoniae* by culturing. The culture positive rate was higher in

under 14 years of age children. **Serotyping:** In this study 15 serotypes were identified and serotype 1(21%) was the leading cause of pneumococcal infection, followed by serotypes 19F and 20 (each 10%) and serotype 14 (8%). Out of the serotypes identified, four serotypes were PCV-10 and five serotypes were PCV-13 vaccine serotypes (Table 2).

Antimicrobial susceptibility: Out of the 68 pneumococcal isolates tested, 44 (64.7%) and 24 (35.3%) were found to be susceptible and intermediate

resistant to penicillin respectively. All but one pneumococcal isolates were susceptible to ceftriaxone (Table 3).

Table 2: Serotype distribution for characterization of pneumococcal isolates from patients in Ethiopia, June to December 2012.

Serotype	Frequency	Percent
1	14	21
19F	7	10
20	7	10
14	6	8
27	6	8
NT	5	6
19A	3	4
18F	3	4
5	3	4
7C	3	4
8	3	4
10A	2	3
13	2	3
22A	2	3
15C	1	2
46	1	2

Table 3: Antibiotics susceptibility pattern of pneumococcal isolates from patients in Ethiopia, June to December 2012.

Antibiotics	Susceptible	Intermediate	Resistant
Penicillin	44	24	0
Ceftriaxone	67	0	1
Erythromycin	59	0	9
Tetracycline	50	1	17
Chloramphenicol	65	0	3
Trimethoprim- sulphamethoxazole	43	4	21

Multilocus sequence typing results: After summing up all results of the seven housekeeping genes, 17 STs (sequence types) were identified; of which 7 were novel (8873, 8874, 8875, 8876, 8877, 8974 and 8975). Out of the seven multiple serotypes, four of them were the same clone while three of them were different serotypes and different clones (Table 4 and 5).

Real Time PCR results: In this study, 140 cultures negative CSFs were subjected to RT-PCR analysis and 23 of them were positive for bacterial meningitis agents. *N. meningitidis* were the leading agent (n=12) followed by *S. pneumoniae* (n=9) and two samples were positive for multiple agents one with *S.pneumoniae* and *N.meningitidis* and the other for *S.pneumoniae* and *H. influenzae*.

Table 4: Result of sequence typing for characterization of pneumococcal isolates from patients in Ethiopia, June to December 2012.

Sequence type (ST)	Frequency	Serotypes
63	6	14
217	8	1
289	3	5
303	2	1
1203	9	19 F = 7 and NT=2
1475	8	27 = 6 and NT= 2
2054	3	7C
2345	3	19A
2711	4	18F = 3 and NT = 1
6451	7	20
8873	2	22A
8874	3	8
8875	2	13
8876	2	10A
8877	4	1
8974	1	15C
8975	1	46

Table 5: Serotypes versus Multilocus sequence typing of pneumococcal isolates from patients in Ethiopia, June to December 2012.

Serotype	ST	aroE	Gdh	Allelic profile				
				gkl	recP	spi	Xpt	Ddl
1	217	10	18	4	1	7	19	9
	303	10	5	4	1	7	19	9
	8877	10	5	4	1	376	4	94
5	289	16	12	9	1	41	33	33
7C	2054	7	13	4	3	9	6	8
8	8874	2	5	9	1	125	14	18
10A	8876	2	19	2	181	7	22	9
13	8875	2	16	19	1	9	155	8
14	63	2	5	36	12	17	21	14
15C	8974	7	42	2	1	1	1	20
18F	2711	10	13	19	5	15	1	145
19F	1203	10	41	47	63	6	14	2
19A	2345	2	13	8	6	25	6	14
20	6451	1	2	29	4	43	1	255
22A	8873	13	2	9	1	6	1	14
27	1475	2	17	107	24	9	105	4
46	8975	43	5	1	1	103	20	70
Non typable	1203	10	41	47	63	6	14	2
Non typable	1475	2	17	107	24	9	105	4
Non typable	2711	10	13	19	5	15	1	145

Discussion

The general culture positive rate in this study was around 27% (61/228). The culture detection rate was low and it might be due to other confounding factors such as prior antibiotic treatment and non-bacterial meningitis since this study used left over samples which could not be possible to obtain detail clinical information that could likely explain the observed low culture positive rate (Greiner et al. 2001). Beside this, most of the CSF samples were clear indicating non pyogenic meningitis and all meningitis agents other than bacteria were not addressed in this study. The rate of pneumococcal isolation is seasonal and collecting samples beyond the pick season also may reduce its yield. Culture negativity among infant patients up to the age of 12 months might be in part due to the introduction of PCV-10 vaccine in Ethiopia a year before the study started. In line with the current study, a report from Israel also documented that the introduction of 9-valent vaccine was found to be associated with a decrease in the carriage rate (Chanteau et al. 2006).

Penicillin G is the primary choice for empirical treatment of pneumococcal infections. In the present study, despite none of the isolates were resistant, 35% (24/68) of them were intermediate resistant for penicillin which could be an alarming sign. Significant increase in bactericidal resistance was also documented in studies conducted in Ethiopia and other African countries (Adegobola et al 2006; Dagan et al. 2002).

In this study, a total of 15 serotypes were identified including non typable isolates. Serotype 1 was the leading cause of pneumococcal infection followed by 19F and 20 serotypes. This finding is in agreement with similar studies conducted in the Gambia (Adegobola et al 2006), Kenya (Dagan et al. 2002) and other studies done in different African countries that showed serotype 1 to be the leading cause of pneumococcal infection in the continent (Assefa et al. 2013; Scott et al. 1998). Together with 19F isolate, one non-typable isolate was identified from the CSF sample. Both isolates (19F and NT) have the same ST and this might indicate that the organism could have lost its capsule and become non typable (Scott et al. 1998). But other serotypes (6A, 6B, 23F) that are most commonly reported in the above studies were not encountered in the present study.

MLST is used to identify the similarities between isolates using housekeeping genes which are common for all clinical isolates. The data is used to classify pneumococcal isolates according to the degree of relation among serotypes for molecular epidemiology. One new allele which found in this study was spi (376).

Real Time-PCR has a high sensitivity and specificity in identifying bacterial pathogens from clinical samples. The method has high sensitivity detecting up to a single DNA molecule (Scott et al. 1998; Mudhune et al. 2009).

In this study, 140 culture negative CSF were tested for the presence of three bacterial pathogens, *N.meningitidis*, *H.influenzae* and *S.pneumoniae*. Out of 140 culture negative CSF samples, 23 were positive for meningitis associated agents and two of them were mixed agents. Identifying mixed organisms from the very sterile sites is rare and it might be due to contamination during sample collection or laboratory analysis. Unable to run the rest of samples using RT-PCR was the limitation of the current study.

Conclusion

The finding from the present study shows that multiple serotypes of *S. pneumoniae* were identified with serotype 1 being the leading. Seven novel sequence types were observed for the international pneumococcal data base. This new data has a significant contribution on STs from a country with no previous data. This initiates to perform another enhanced pneumococcal surveillance with large number of samples. Antimicrobial susceptibility of isolates in the health facilities should be checked in microbiology laboratory prior to empirical treatment. In order to decrease false negative results, sensitive laboratory tools such as Real-Time PCR should be part of diagnosis at least at the referral level.

Competing interests: The authors declare that they have no competing interests.

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