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ORIGINAL RESEARCH

Serotype Distribution of *Streptococcus pneumoniae* Isolates Causing Invasive and Non-Invasive Infections Using Whole-Genome Sequencing in Ethiopia

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Background: In Ethiopia, pneumococcal conjugate vaccine 10 (PCV10) was introduced in 2011 in the national vaccination program. This study was aimed to assess serotype distribution of invasive and non-invasive *Streptococcus pneumoniae* isolates using whole-genome sequencing.

Methods: A hospital-based prospective study was conducted from 2018 to 2019 at Addis Ababa and Amhara region referral hospitals, from all patients. Clinical Samples were collected and initially cultured onto 5% sheep blood agar at 37°C in a 5% CO_2 atmosphere. Sequencing was done using the Illumina NextSeq 500 and SeroBA was used to predict serotypes from whole-genome sequencing raw data.

Results: Of the 57 *S. pneumoniae* isolates, there were 32 circulating serotypes. The most common serotypes were 15A/B/C (n=5, 8.8%), 6A (n=4, 7.0%), 10A/F (n=4, 7.0%), 23A (n=4, 7.0%) and 7C (n=3, 5.3%). The serotype coverage of PCV10 and PCV13 were 12.3% and 26.3% respectively. The most common invasive serotypes were 15A/B/C (n=5, 8.8%) and 6A (n=4, 7.0%), and non-invasive serotypes were 23A (n=4, 7.0%) and 10A/F (n=3, 5.3%). The most prevalent serotype obtained from PCV10 eligible children was 3 (n=2, 3.5%). The prevalent serotype obtained from PCV10 non-eligible patients were type 23A (n=4, 7%) and type 6A (n=3, 5.2%). The most common serotypes among children ≤18 years old were 10A/F, 7C, 35A/B, 16F, 19A, 3 and 38. However, the proportions of some non-vaccine serotypes (11A/B and 15A/B/C) were higher in adult patients.

Conclusion: In this study a shift in the distribution of non-vaccinated *S. pneumoniae* serotypes increases in the population, and PCV10 serotype coverage was reduced as compared to PCV13. Therefore, it is important to continue monitoring serotype changes among all patients in addition to assessing the impact and effectiveness brought by vaccines and provides a foundation for prevention strategies and vaccine policies.

Keywords: *Streptococcus pneumoniae*, serotypes, whole-genome sequencing

Introduction

Streptococcus pneumoniae is a major pathogen that causes community-acquired pneumonia (CAP), acute exacerbations of chronic bronchitis, meningitis, sinusitis, otitis media, and sepsis; which are divided into invasive and non-invasive infections.[1] Isolates of *S. pneumoniae* are traditionally characterized in terms of the antigenicity of their capsular polysaccharides (CPS) and to date, there are nearly

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100 known serotypes described for *S. pneumoniae* based on the differences in the antigenic properties of the capsule.[2]

Serotyping methods of *S. pneumoniae* can be grouped into two different categories: phenotype-based methods and genotype-based methods.[3] The phenotype-based methods (based on antisera reactions) remain the Gold Standard method used in most laboratories.[4] However, this method is expensive, lengthy, and not fully reliable. Genotype-based methods were developed to provide cost-effective and reliable assays for the serotyping of *S. pneumoniae*.[3,5] Among these methods, Whole-genome sequencing (WGS) became a suitable method for serotyping with the improvement in accuracy and a decrease in cost which has allowed the identification of serotype by comparing cps loci sequences.[6–9]

Serotype surveillance projects around the world showed an increase in *S. pneumoniae* disease due to non-vaccine serotypes that are caused by serotype replacement.[10–14] Therefore, it is very important to survey the circulating serotypes, to observe the epidemiological trends of *S. pneumoniae* before and after vaccination. Globally, serotypes 19A (28%), 19F (10%), and 14 (9%) were the most common in children under 5 years. In adults over 16 years, 19A (13%), 3, 6A and 7F (all 7%) were most common.[15]

A higher number of serotypes are responsible for an equal proportion of invasive and non-invasive pneumococcal diseases in Africa and Asia than in North America, suggesting a greater diversity of serotypes causing disease in Africa and Asia continents.[16] As a result, the World health organization (WHO) recommended the inclusion of pneumococcal conjugate vaccines in national immunization programs throughout the world.[17]

In Ethiopia, Pneumococcal conjugate vaccine 10 (PCV10) was introduced in 2011 in the national vaccination program as a 3 dose primary series at 6, 10, and 14 weeks of age and no booster dose (3p + 0).[18] The coverage of PCV10 in Ethiopia is 63% by WHO and UNICEF and 95% by Ethiopian Minister of Health estimates of immunization coverage, 2019 report.[19] Although the serotypes that circulate in different communities were not known only two studies have previously reported on the prevalence of pneumococcal and *Haemophilus* serotypes.[20,21] These studies, however, had limitations in which the sample sources were CSF using the quellung method and the target population was children only and also one study performed two decades ago. So, more recent studies with

an advanced method of characterization from different sample sources are mandatory to generalize the serotype distribution of *S. pneumoniae* for the entire country.

Information on serotype distribution is important to change the current PCV10 to more vaccine number coverage of PCVs. Therefore, the main goal of this study was to assess the serotype distribution of *S. pneumoniae* isolates which are responsible for invasive and non-invasive infections in children and adults from clinical isolates of suspected pneumococcal infections in Ethiopia using WGS assay.

Methods
Patients and Clinical Isolates

A hospital-based prospective study was conducted from January 2018 to 2019 at Addis Ababa (Yekatite12 Hospital, Alert Hospital, and International Clinical Private Laboratory) and Amhara Region (University of Gondar Comprehensive Specialized Hospital, Felege Hiwot Comprehensive Specialized Hospital and Dessie Regional Laboratory) Referral Hospitals, Ethiopia. During the study period, a total of 70 phenotypically confirmed *S. pneumoniae* were isolated from pediatric and adult patients suspected of pneumococcal infections. Clinical samples (cerebrospinal fluid, blood, sputum, eye discharge, ear discharge, pleural and peritoneal fluids) were collected from appropriate collection sites and were initially cultured onto 5% sheep blood agar plates overnight at 37°C in a 5% CO_2 atmosphere. *S. pneumoniae* was identified and confirmed by typical colony morphology, alpha-hemolysis, Gram staining, optochin susceptibility, and bile solubility test. The strains were stocked in Skimmed Milk-Trypticase Soy-Glucose-Glycerol (STGG) medium and transported to Norwegian Institute of Public Health Microbiology Laboratory, Oslo, Norway using dry ice for serotyping using WGS.

PCV status was determined on the base of patients' age at the date when the pneumococcal strain was isolated. Each patient was categorized according to his/her immunization status at pneumococcal disease presentation as either "PCV10 vaccinated" or "PCV10 non-vaccinated". All children born on September 1st, 2011, or thereafter were eligible for vaccination regardless of whether they had received the vaccine or not. We defined them as a group of vaccinated children who had received 3 doses of PCV10. Another group of children born less than 14 weeks of age and adults not eligible for PCV10

vaccination (those born before September 2011) from the data recorded on the request of cultures to laboratories was defined as non-vaccinated individuals. We compared serotype distribution rates for both groups of patients (PCV10 eligible children and PCV10 non-eligible children and adults).

DNA Extraction of *Streptococcus pneumoniae*

Isolates were recovered on a blood agar plate with 5% sheep blood and incubated overnight at 37°C in a 5% CO_2 atmosphere. Bacteria were collected with two full inoculating loops and suspended in the ATL master mix. Samples were then frozen at −20°C until extraction. DNA extraction was performed with the QIAamp@DNA Mini Kit, QIAGEN, and QIAcubeTM BioRobot machine work station according to the manufacturer's instructions. DNA concentrations were measured using Qubit 4 fluorometer and QubitTM 1X dsDNA HS Assay Kit (Thermo Fisher Scientific); 40 ng of genomic DNA was used for sample preparation.

Pneumococcal Serotyping

Pneumococcal serotypes/groups were determined for the 70 isolates using WGS. Based on the WGS result, 57 (81.4%) isolates were further confirmed as *S. pneumoniae*. Libraries for WGS were prepared with the KAPA HyperPlus DNA library preparation kit and sequenced using an Illumina MiSeq reagent kit v3 (600 cycles, paired ends) following the manufacturer's instructions. Illumina Nextera DNA libraries were constructed and sequenced using the Illumina NextSeq 500. SeroBA was used to predict serotypes, by identifying the cps locus, directly from raw whole genome sequencing read data with 98% concordance using a kmer-based method, can process 10, 000 samples in just over 1 day using a standard server, and can call serotypes at coverage as low as 15–21X. SeroBA is implemented in Python3 and is freely available under an open-source GPLv3 license from https://github.com/sanger-patho gens/seroba.[6] If the serotype was not detected by the method mentioned above, the strain was classified as non-typable due to the lack of a capsular operon. Afterward, the coverage rates of PCV10 and PCV13 were estimated by calculating the percentage of isolates expressed the serotypes included in the vaccines.

Data Analysis

Data were entered and analyzed using the Statistical Package for the Social Science (version 20; SPSS Inc, Chicago, IL, USA). The rates of serotype distribution among vaccinated and unvaccinated patients, source of samples, and age group were compared using tables and figures. Discrete variables were expressed as percentages and proportions.

Results
Demographic and Clinical Data of Patients

We analyzed 70 phenotypically confirmed *S. pneumoniae* isolates recovered from specimens of children and adults with invasive and non-invasive infections. However, using whole-genome sequencing method, 57 (81.4%) isolates were *S. pneumoniae*, 6 (8.6%) were *S. mitis*, 1 (1.4%) was *S. oralis*, 1 (1.4%) was *S. peroris* and 1 (1.4%) was *Enterococcus avium*. Among 57 *S. pneumoniae* isolates, 37 (65%) were obtained from children aged between 38 days to 18 years; the remaining 20 strains (35%) were recovered from adult patients. Out of the 37 children, 20 had received PCV10 vaccination (age-based vaccine status), covering 35.1% out of all patients with invasive and non-invasive infection (Table 1). Invasive disease refers to isolation of *S. pneumoniae* from a normally sterile body sites like blood, cerebrospinal fluid (CSF) or pleural fluid and non-invasive pneumococcal disease included non-bacteremic pneumonia cases and those with isolates from non-sterile sites such as sputum, middle ear fluid and eye discharge. The clinical sources of isolates were cerebrospinal fluid (CSF) 20 (35.1%), blood 12 (21.1%), eye discharge 10 (17.5%), sputum 10 (17.5%), pleural fluid 2 (3.5%), ear discharge 2 (3.5%) and peritoneal fluid 1 (1.8%).

Serotype Distribution and Conjugate Vaccine Coverage of *S. pneumoniae* Isolates

Serotype distribution of both invasive and non-invasive isolates according to source of the strain isolation, vaccine status and age group is presented in Table 1. There were thirty-two (32) circulating serotypes amongst the isolates: 18 were found in invasive isolates only, 10 from non-invasive isolates only and 4 from both invasive and non-invasive isolate (Figures 1 and 2). Generally, the most

Table 1 Distribution of *S. pneumoniae* Serotypes According to Source of Strain Isolation, Vaccination Status and Age Group at Addis Ababa and Amhara Region Referral Hospitals, Ethiopia, 2018–2019

Serotype/Serogroup(s)	Total No. (%) of Particular Serotype	% of Serotype by Source		% of Serotype by Vaccine Status		% of Serotype by Age Group	
		Invasive	Non-Invasive	PCV10 Eligible Children	PCV10 Non-Eligible Patients	Children	Adults
15A/B/C[c]	5 (8.8)	8.8	0.0	3.5	5.3	3.5	5.3
6A	4 (7.0)	7.0	0.0	1.8	5.2	3.5	3.5
10A/F[d]	4 (7.0)	1.7	5.3	1.8	5.2	5.2	1.8
23A	4 (7.0)	0.0	7.0	0.0	7.0	3.5	3.5
7C	3 (5.3)	3.5	1.8	1.8	3.5	3.5	1.8
11A/B[e]	3 (5.3)	1.8	3.5	0.0	5.5	1.8	3.5
13, 20, 21[a]	3 (5.3)	0.0	5.3	0.0	5.5	0.0	5.3
35A/B[f]	3 (5.3)	1.8	3.5	1.8	3.5	5.3	0.0
NT[b]	3 (5.3)	3.5	1.8	5.3	0.0	5.3	0.0
8	2 (3.5)	3.5	0.0	0.0	3.5	1.8	1.8
14	2 (3.5)	3.5	0.0	1.8	1.8	1.8	1.8
16F	2 (3.5)	3.5	0.0	0.0	3.5	3.5	0.0
17F	2 (3.5)	0.0	3.5	1.8	1.8	1.8	1.8
19A	2 (3.5)	0.0	3.5	1.8	1.8	3.5	0.0
24, 29[a]	2 (3.5)	1.8	1.8	3.5	0.0	3.5	0.0
1	2 (3.5)	3.5	0.0	0.0	3.5	1.8	1.8
3	2 (3.5)	3.5	0.0	3.5	0.0	3.5	0.0
38	2 (3.5)	1.8	1.8	0.0	3.5	3.5	0.0
45, 46[a]	2 (3.5)	3.5	0.0	1.8	1.8	1.8	1.8
7F	1 (1.8)	1.8	0.0	0.0	1.8	1.8	0.0
18A	1 (1.8)	1.8	0.0	1.8	0.0	1.8	0.0
19F	1 (1.8)	1.8	0.0	0.0	1.8	0.0	1.8
23F	1 (1.8)	1.8	0.0	1.8	0.0	1.8	0.0
41F	1 (1.8)	0.0	1.8	1.8	0.0	1.8	0.0
Total No. (% of vaccinated children)	57 (35.1)	35 (40.0)	22 (27.3)	20 (100)	37 (0.0)	37 (54.1)	20 (0.0)
% of PCV10 vaccine coverage	12.3	20.0	0.0	10.0	13.5	10.8	15.0
% of PCV13 vaccine coverage	26.3	37.1	9.1	30.0	24.3	27.0	25.0

Notes: [a]Expected to serotypes/serogroups (strains were positive with one of the pooled sera only). [b]Non-typable strains. [c]Serogroup 15 included serotypes: 15A (= 1); 15B (= 3); and 15C (= 1). [d]Serogroup 10 included serotypes: 10A (= 3) and 10F (= 1). [e]Serogroup 11 included serotypes: 11A (= 1) and 11B (= 2). [f]Serogroup 35 included serotypes: 35A (= 1) and 35B (= 2).

common serotypes were 15A/B/C (n=5, 8.8%), 6A (n=4, 7.0%), 10A/F (n=4, 7.0%), 23A (n=4, 7.0%) and 7C (n=3, 5.3%). All of these serotypes, except type 6A, are non-vaccine serotypes. In the whole isolate collection, the serotype coverage of PCV10 and PCV13 were 12.3% (n= 7) and 26.3% (n = 15) respectively (Table 1).

Depending of source of pneumococcal isolates, the most common serotypes among invasive cases were 15A/B/C (n=5, 8.8%), 6A (n=4, 7.0%) 7C, 8, 14, 16F, 1 and 3 (n=2, 3.5% each). The PCV10 and PCV13 vaccine coverage rates were 7 (20.0%) and 13 (37.1%) for patients with invasive cases, respectively. Out of the 35 invasive

isolates, fourteen were obtained from PCV10 eligible children (40%). Among PCV10 eligible children, seven were from vaccine serotypes (14, 19F, 23F, 1, 7F, 3, 6A) of both PCV10 and PCV13. However, the most common serotypes among non-invasive cases were 23A (n=4, 7.0%), 10A/F (n=3, 5.3%), 11A/B, 35A/B, 17F, and 19A (n=2, 3.5% each), and the vaccine coverage rate of PCV13 was 9.1% but not vaccine coverage for PCV10. Out of the 22 non-invasive isolates, six were obtained from PCV10 eligible children (27.3%). Among them, one was vaccine serotypes 19A. The proportions of vaccinated children among 35 invasive *S. pneumoniae* isolates were higher (40.0%)
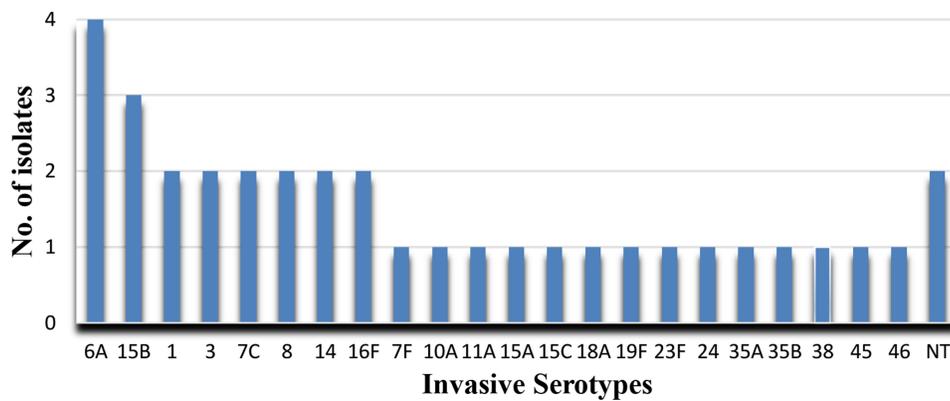
Figure 1 Distribution of *S. pneumoniae* serotypes isolated from invasive pneumococcal diseases at Addis Ababa and Amhara Region Referral Hospitals, Ethiopia, 2018–2019.
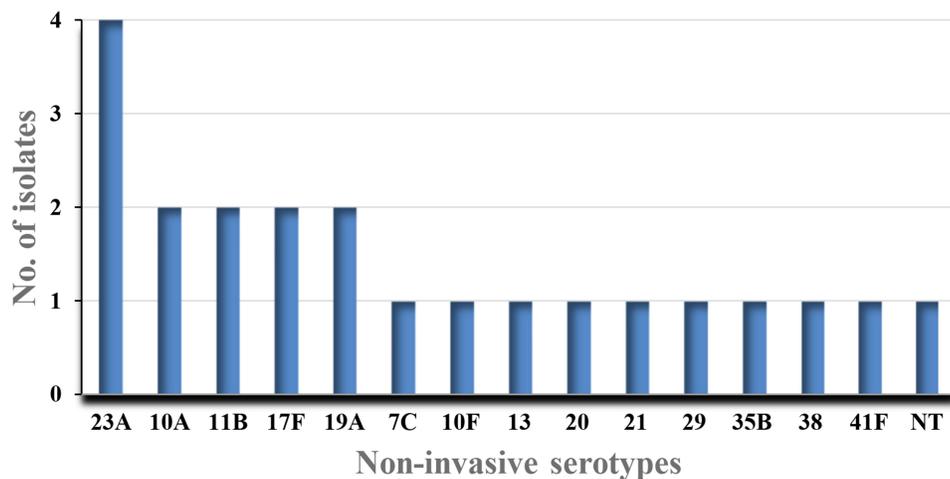
Figure 2 Distribution of *Streptococcus pneumoniae* serotypes isolated from non-invasive pneumococcal diseases at Addis Ababa and Amhara Region Referral Hospitals, Ethiopia, 2018–2019.

than the proportion of vaccinated children with non-invasive strains (27.3%).

Serotypes distribution of the isolates among the study population was also analyzed according to the vaccination status of the study participants. In isolates obtained from PCV10 eligible children (n = 20), the most prevalent serotype was 3 (n=2, 3.5%). In this group of vaccinated eligible children, 2 (10%) and 6 (30%) of the serotypes were vaccine serotypes included in PCV10 and PCV13, respectively. Among pneumococci obtained from PCV10 non-eligible patients with invasive and non-invasive isolates (n = 37), the most prevalent serotypes were 23A (n=4, 7.0%), 6A (n=3, 5.2%), 10A, 7C, 15B, 11B, 35B, 8, 16F, 1 and 38 (n=2, 3.5% each). In this group of non-eligible patients, PCV10 and PCV13 were 13.5% and 24.3% serotype coverages, respectively. The proportions of some non-vaccine serotypes (15A/B/C, 23A, 38, 16F, 8, 11A/B, 7C, 10A/F) were higher in

PCV10 non-eligible patients than in the PCV10 eligible children. Similarly, some vaccine serotype isolates (6A, 1) were more often found in the non-eligible patients than in the eligible ones. However, only one vaccine serotype (3) showed higher rates in PCV10 eligible children.

Another serotype distribution in the study population was analyzed according to age groups. The most common serotypes among children ≤18 years old were 10A/F, 7C, 35A/B, 16F, 19A, 3, and 38. Serotype 19A and 3 were included in PCV13 but not included in PCV10, the vaccine currently included in the national vaccination program in Ethiopia. In this group of children, PCV10 and PCV13 were 10.8% and 27.0% serotype coverages, respectively. However, the proportions of some non-vaccine serotypes (11A/B and 15A/B/C) were higher in adult patients. The vaccine coverage rate of PCV10 and PCV13 in adults was 15% and 25%, respectively.

Discussion

S. pneumoniae serotyping has become more important since the release of the different vaccines for the monitoring of emerging non-vaccine serotypes. However, the information on pneumococcal serotype might not directly benefit the clinical diagnosis or the immediate treatment of the patient. The knowledge of pneumococcal serotypes circulating in carriage and disease is required to correctly estimate the impact of pneumococcal vaccines locally and globally and is needed by vaccine policymakers. In this study, we present serotype-specific variant profiles that can be used to distinguish 32/57 serologically distinct serogroups/serotypes and SeroBA, a bioinformatics tool that uses the capsular typing variant database to predict capsular type from WGS raw data.

Our present study demonstrated that the most common serotypes were 15A/B/C, 6A, 10A/F, 23A, and 7C, and the serotype coverages of PCV10 and PCV13 were 12.3% and 26.3%, respectively. All of these serotypes, except type 6A, were non-vaccine serotypes. Which was similar to other recent studies in Malawi,[7] Russia,[8] Germany,[13] Japan,[22] and China[23] but the ranking orders varied. Vaccine coverage was lower in our study compared to other countries. In other words, there is a change in the relative rates of the most common serotypes observed due to a reduction of PCV10/13 vaccine types and a concurrent increase in non-vaccine serotypes. Because pneumococcal vaccine was included in the National vaccination schedule in September 2011 and all of the serotypes were not included in the PCV10 vaccine.

In this study, serotype 15A/B/C was isolated most frequently among all invasive isolates, followed by types 6A, 7C, 8, 14, 16F, 1, and 3. Similar findings have reported in recent studies.[7,8,13,14,23,24] However, marked differences between countries, with serotype 15A/B/C dominating in Germany and China, serotype 6A dominating in China and Russia, serotype 7C dominating in China, and England and wales, serotype 3 dominating in Russia, and serotype 1 dominating in Malawi and other 11 African countries. The proportion of strains with serotypes included in PCV10 and PCV13 vaccines was 20% and 37.1%, respectively. Because five isolates were included in PCV10 and seven isolates were included in PCV13.

The most common serotypes among non-invasive isolates were 23A, 10A/F, 11A/B, 35A/B, 17F, and 19A, which is consistent with other countries in the world.[7,22] The vaccine coverage rate of PCV13 was 9.1% but zero coverage rates for PCV10. This is because only six isolates were received from vaccinated children and the serotypes were not included in PCV10, only one serotype (19A) was included in PCV13.

Also, serotype 3 was more prevalent among vaccinated children with vaccine coverage of 10% for PCV10, and 30% for PCV13. Serotype 23A, 6A, 10A, 7C, 15B, 11B, 35B, 8, 16F, 1 and 38 were the most prevalent serotypes among non-vaccinated patients with a vaccine coverage of 13.5% for PCV10 and 24.3% for PCV13. As expected we observed that the introduction of vaccine was followed by a decrease in the rate of vaccine serotype accompanied by an increase in the diversity of non-vaccine serotype for both vaccinated and non-vaccinated individuals,[25] a result that is in line with findings in other countries.[7,8,23,24]

In the present study, we reported the increase of 10A/F, 7C, 35A/B, 16F, 19A, 3, and 38 serotypes prevalence among pediatrics (≤ 18 years of age) and 11A/B and 15A/B/C were more prevalent among adults (> 18 years old). The vaccine coverage of pediatrics was 10.8% for PCV10 and 27% for PCV13, and 15% for PCV10 and 25% for PCV13 among adults. Our findings similar to the findings of other studies done in other countries with a rank difference like serotype 3 and 38 dominating in Russia,[8] 7C dominating in England and Wales,[14] 10A dominating in Japan,[22] and 15A/B/C dominating in China.[23] After the introduction of PCV10 into our childhood immunization program, the majority of the circulating pneumococci among vaccine eligible children were non-vaccine type which is included in PCV13 such as serotype 6A, 3, and 19A.

Compared to global data and local studies, our results demonstrated that in the total pneumococcal isolates studied, a serotype replacement was observed following the introduction of PCV10. Overall, serotype 6A appeared as the most common vaccinated serotype among all patients, and serotype 23A, 15B, 10A, and 7C also appeared as the most common non-vaccinated serotypes among all patients. Vaccine coverage was lower in our study as compared to other countries mentioned above. Although the vaccine is not widely used, we still observed the phenomenon of serotype changes, which suggests that the changes in serotype may be directly related to vaccination (serotype replacement)[10,12,14] or not directly related to vaccination (pneumococci was able to change their capsular serotype by exchanging the capsular locus genes).[26] The limitation of this study is the small sample size, and also lake of pre-vaccination

data, descriptive study and not a vaccine effectiveness study in Ethiopia. We will perform continuous surveillance in Ethiopia to draw a reasonable analysis of the distribution of serotypes causing invasive and non-invasive infections.

Conclusions

Vaccination has substantially reduced vaccine serotype pneumococcal invasive and non-invasive diseases among all patients' ages after the introduction of PCV10 as a routine vaccine immunization program. However, a shift in the distribution of non-vaccinated *S. pneumoniae* serotypes increases in the population, and PCV10 serotype coverage was reduced as compared to PCV13. Therefore, it is important to continue monitoring serotype changes among all patients in addition to assessing the impact and effectiveness brought by vaccines and provides a foundation for prevention strategies and vaccine policies.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

All procedures performed in this study were reviewed and approved by the ethical review board of the University of Gondar (No. O/V/P/RCS/05/377/2017) in accordance with the 1964 Helsinki declaration. Permission was obtained from each hospital laboratory for collecting the isolates. Written informed consent was obtained from all patients, and a parent or legal guardian of patients under the age of 18 years after explaining the purpose and objective of the study.

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Author Contributions

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Disclosure

The authors declare that they have no competing interests for the publication of this paper.

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