ORIGINAL RESEARCH

Phenotypic and Molecular Characterization of Penicillin and Macrolide-Resistant *Streptococcus pneumoniae* Serotypes Among Pediatric Patients in Addis Ababa, Ethiopia

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Background: In several countries, introduction of the pneumococcal conjugate vaccine (PCV) has led to a decline in antimicrobial-resistant pneumococcal disease but has also resulted in a concomitant increase in antimicrobial-resistant, non-vaccine serotypes of *Streptococcus pneumoniae*. We sought to determine the magnitude of penicillin and macrolide resistance among pneumococcal serotypes and the mechanisms of macrolide resistance in Ethiopia, 5 years after the introduction of PCV10 in the country.

Methods: Susceptibility to penicillin and erythromycin of 119 pneumococcal isolates collected from pediatric patients aged 0–15 years in Addis Ababa, Ethiopia, was tested using disc diffusion, and minimum inhibitory concentration (MIC) was also determined by Etest. Pneumococcal serotypes were determined by sequencing the *cps*B gene and using Quellung reaction. Polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism analysis were used to detect and differentiate the macrolide resistance genes *erm*(B), *mef*(A), and *mef*(B).

Results: Among the 119 isolates, 2.5% (3/119) were resistant to penicillin, while 58% (69/119) were intermediate. Resistance to erythromycin was observed in 33.6% (40/119) of the isolates with the highest level of resistance among isolates from middle ear discharge, i.e., 53.3% (8/15). Half (19/40) of the erythromycin resistant isolates were serotype 19A and among serotype 19A isolates, the majority i.e., 54.3% (19/35) were resistant to erythromycin. The most common macrolide resistance determinant was *mef*(E) with a prevalence of 50% (20/40).

Conclusion: Five years after introduction of PCV10 in Ethiopia, we observed that the prevalence of penicillin-resistant *S. pneumoniae* was low. However, there was a high level of macrolide resistance which was mostly in serotype 19A, and the resistance was mainly mediated by efflux pumps. Introduction of PCV13 (which covers serotype 19A) would significantly improve coverage of the macrolide-resistant serotypes. Continued surveillance of pneumococcal serotype distribution and their antibiotic resistance pattern in Ethiopia is warranted.

Keywords: efflux pump, erythromycin, *mef*(E), PCV10, penicillin, serotype 19A, *Streptococcus pneumoniae*

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Introduction

Streptococcus pneumoniae (pneumococcus) still remains the major cause of pneumonia, meningitis, bacteremia and otitis media among infants and young children, worldwide. A 2015 estimate indicates that globally, there were 294,000 deaths due to *S. pneumoniae* in children aged 1–59 months.[1]

Penicillin non-susceptible pneumococci (PNSP) are on the priority list of antibiotic-resistant bacteria listed by the WHO for which research and development of new antibiotics are required.[2] PNSP were first detected soon after the discovery of penicillin[3] and have since spread globally.[4] A recent meta-analysis of antimicrobial susceptibility profiles for pneumococcal pneumonia in sub-Saharan Africa indicated that the susceptibility to penicillin was 68.6%.[5]

Although the increasing prevalence of macrolide-resistant *S. pneumoniae* is raising concerns on the use of macrolides for treating pneumococcal diseases, they still remain an important group of antimicrobials.[6] In the Ethiopian standard treatment guideline, macrolides are the first alternatives to β-lactams.[7] Previous reports from Ethiopia indicate higher resistance to macrolides (23.9%) compared with other antibiotics (15.0% for penicillin and 20.4% for amoxicillin), in nasopharyngeal pneumococcal isolates[8] and a macrolide-resistance prevalence of 44.4% among isolates causing otitis media.[9]

Before the introduction of PCVs, the pneumococcal serotypes with highest level of resistance to penicillin and erythromycin were 6B, 6A, 9V, 14, 15A, 19F, 19A, and 23F, globally.[10] After introduction of PCV7 in the vaccination schedule of various countries in the 2000s and higher valent PCV10 afterwards, there was a significant decline in antimicrobial-resistant pneumococcal carriage and disease due to vaccine serotypes. This effect was however offset by the increasing resistance in non-vaccine serotypes, specifically serotype 19A.[11]

PCV10 was introduced in Ethiopia in October 2011 as a three dose primary series (3p+0) without any booster dose.[12] The recent Ethiopian health and demographic survey indicated that 70.1% children aged < 3 years surveyed from the whole country and 93.1% of those from Addis Ababa received all three doses of PCV10.[13] Most of the previous studies on penicillin resistance in *S. pneumoniae* in Ethiopia[8,9] used the oxacillin disc diffusion test as a proxy for penicillin resistance and therefore lack accuracy. The CLSI criteria state that for isolates with oxacillin zones ≤ 19 mm, penicillin resistance should not be reported without performing a penicillin MIC test.[14] In addition, there is a lack of data on the prevalence and distribution of PNSP serotypes in the post-PCV10 era.

In *S. pneumoniae*, macrolide resistance is mainly mediated by two mechanisms. The first one, target-site modification by erythromycin methylase (*erm*(B)) gene, usually confers high-level resistance and cross-resistance to lincosamides and streptogramin B drugs (MLS$_B$ phenotype). The second mechanism is mediated by *mef* class of genes that lead to active drug efflux and results in low- to mid-level resistance to macrolides (M phenotype). The *mef* gene has two variants, *mef*(A), originally found in *Streptococcus pyogenes*, and *mef* (E), which was first described in *S. pneumoniae*.[15] The two *mef* genes have around 90% sequence homology and can be distinguished using specific primer sets. Due to the sequence similarity between the two genes, they were merged under *mef*(A) by Roberts and colleagues.[16] In Ethiopia, in a study performed before the introduction of PCV10, the most common macrolide resistance determinant identified was *mef*(A/ E).[17] There are however no data on the magnitude and molecular mechanisms of macrolide resistance among different pneumococcal serotypes in the post-PCV10 era in Ethiopia. Therefore, the aim of this study was to determine the magnitude of penicillin and macrolide resistance among pneumococcal serotypes and the mechanisms of macrolide resistance in Addis Ababa, Ethiopia, 5 years after the introduction of PCV10 in the country.

Materials and Methods
Study Sites and Source of Bacterial Isolates
The pneumococcal isolates were collected from September 2016–August 2017 from children aged 0–15 years attending pediatric emergency departments at Black Lion and Yekatit 12 hospitals and pediatric outpatient departments in Girum hospital and Dr. Yared Pediatric Specialty Center, in Addis Ababa, Ethiopia. A total of 119 *S. pneumoniae* isolates were collected, comprising 89 from nasopharyngeal swabs of children with community acquired pneumonia, 17 from nasopharyngeal swabs of children with non-respiratory illnesses, 5 from blood of children with pneumonia (n = 1) or sepsis (n = 4) and 15 from middle ear swabs of children with acute otitis media. Serotypes of most of the pneumococcal isolates (except of the 17 isolates from nasopharyngeal swabs of children with non-respiratory illnesses) were previously determined and have been reported elsewhere.[18–21]

Antimicrobial Susceptibility Testing
Susceptibility to penicillin was initially determined using the Kirby-Bauer disk diffusion method.[22] Oxacillin discs (1 μg) were initially used and for isolates with zones of ≤ 19 mm, the minimum inhibitory concentrations (MICs) were determined using Etest strips (bioMérieux, Marcy-l'Étoile, France). The results were classified as: susceptible

(≤ 0.06 µg/mL), intermediate (0.12–1 µg/mL), and resistant (≥ 2 µg/mL). To determine macrolide resistance, MICs were determined using Etest strips and results were classified as susceptible (MIC ≤ 0.25 µg/mL), intermediate (0.5 µg/mL), and resistant (≥ 1 µg/mL). Test results of both disc diffusion and MICs were interpreted according to the Clinical and Laboratory Standard Institute (CLSI) criteria.[14] American Type Culture Collection (ATCC) strain *S. pneumoniae* ATCC 49619 was used for quality control.

DNA Extraction and Serotyping

DNA from *S. pneumoniae* isolates was extracted using alkaline lysis as previously described.[23] Initial typing of pneumococcal isolates was done using PCRSeqTyping as previously described[24] and consisted of amplification and sequencing of a 1061 bp region of the *cps*B-gene. Sanger sequencing was performed at GATC Biotech (Constance, Germany) using 20 µL of the amplicons. *cps*B sequences were then used to interrogate GenBank database (http://www.ncbi.nlm.nih.gov/blast). BLAST bit score of > 99% sequence identity with reference sequences was used to assign serotypes. Because the correct serotype could not be assigned by PCRSeqTyping for some of the isolates, serotyping was performed on all isolates as well with the Quellung reaction[25] using pool and group antisera, obtained from the Statens Serum Institut (Copenhagen, Denmark).

Detection of *erm*(B) and *mef*(A/E) Genes

PCR was used to detect macrolide resistance genes on all macrolide-resistant (MIC ≥ 1 µg/mL) isolates. Amplification of the ribosomal methylase (*erm*(B)) gene was performed using the primers: *erm*B-F, 5′-GAAAAGGTACTAAACCAAATA-3′ and 5′-AGTAACG GTACTTAAGTTTAC-3′ to generate a 639 bp PCR

product.[26] A PCR-restriction fragment length polymorphism analysis was then performed in order to identify the presence of macrolide efflux genes *mef(*A/E) and discriminate between *mef*(A) and *mef*(E). The primer pair used was: MEF-F, 5′-GCGTTTAAGATAAGCTGGCA-3′ and MEF-R, 5′-CCTGCACCATTTGCTCCTAC-3′, to generate a 1743-bp PCR product. PCR amplification consisted of 35 cycles at 95°C, 54°C, and 72°C for 1 min each. The amplicons were then digested with the BamHI restriction enzyme.[27]

Results
Susceptibility to Penicillin and Erythromycin

The most prevalent serotypes in this study were serotypes 19A (29.4%, 35/119), 16F (9.2%, 11/119), and non-typeables (6.7%, 8/119). PCV10 serotypes constituted 11.7% (14/119) whereas PCV13-unique serotypes constituted 32.7% (39/119). Among the isolates, only 2.5% (3/119) were resistant to penicillin, while 58% (69/119) had intermediate susceptibility and 39% (47/119) were susceptible. The resistant isolates were serotype 19A (n = 2) and non typeable (n = 1). Among the isolates with intermediate susceptibility, most, 40.6% (28/69) were serotype 19A (Figure 1) and among serotype 19A isolates, the majority (80%, 28/35) showed intermediate susceptibility.

Resistance to erythromycin was observed in 33.6% (40/119) of the isolates overall. The highest level of erythromycin resistance was seen among isolates from middle ear discharge, 53.3% (8/15), followed by nasopharyngeal isolates, 32.6% (29/89), and isolates from blood, 20% (1/5) (Table 1). The resistance level to erythromycin by Etest MIC ranged between 3 and > 256 µg/mL, and most (65.8%) of the strains showed a high level of resistance (> 256 µg/mL). All the three isolates that were resistant to
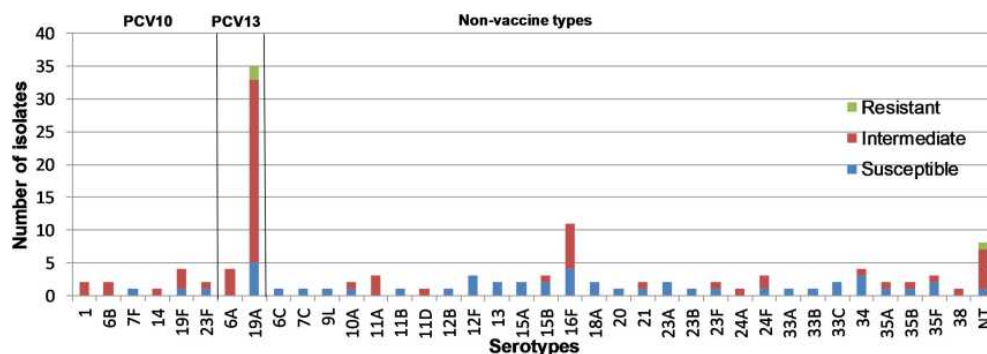

Figure 1 Pneumococcal serotypes from pediatric patients aged 0–15 years in Addis Ababa, Ethiopia and their susceptibility to penicillin.

Table 1 Source Sample, Serotypes and Genotypes of Macrolide-Resistant *S. pneumoniae* Isolates from Pediatric Patients Aged 0–15 Years in Addis Ababa, Ethiopia

Serotype	Total No.	Antimicrobial Susceptibility		Source Sample			Mechanism of Resistance		
		S	R	NP	MES	Blood	*erm(B)*	*mef(A)*	*mef(E)*
1	2	1	1			1	1		1
11A	3	2	1	1					
11D	1		1	1ᵃ					
12B	1		1	1ᵃ					
12F	3	2	1	1				1	
14	1		1	1					
15A	2	1	1	1			1		
15B	3	2	1	1					1
16F	11	8	3	3					1
18A	2	1	1	1					1
20	2	1	1	1					
19A	35	16	19	11	7	1	1		11
23A	2	1	1	1					1
23F	2		2	2ᵃ					
35A	2	1	1	1					1
38	1		1	1					1
NT	8	5	3	2	1				2
Total No.	**81**	**41**	**40**	**30**	**8**	**2**	**3**	**1**	**20**

Note: ᵃIdentified from children with non-respiratory infections.
Abbreviations: S, susceptible; R, resistant; NP, nasopharyngeal swab; MES, middle ear swab.

penicillin were also resistant to erythromycin. Overall, almost half (19/40) of the erythromycin-resistant isolates were serotype 19A and among serotype 19A isolates 54.3% (19/35) were resistant (Figure 2). Among PCV10 serotypes, 6B (2), 7F (1) and 19F (4) were all susceptible to erythromycin, whereas all of serotype 14 (1) and 23F (2) isolates were resistant to erythromycin.

Identification of Macrolide Resistance Determinants

The most common macrolide resistance determinant was *mef* (E), identified in 50% (20/40) of the strains while *mef* (A) was identified in only one strain (2.5%) (Table 1). Among those with *mef* (E), 55% (11/20) were serotype 19A. *Erm*(B) was identified in three strains, two of which

(serotype 1 and 19A) were positive for both *erm*(B) and *mef*(E) and both had a high level of resistance (MIC > 256). Most of the strains which had *mef*(E), 65% (13/20) also showed high level of resistance (MIC > 256). In 40% (16/40) of the resistant erythromycin isolates, neither *erm* (B) nor *mef*(A/E) were detected.

Discussion

Because most antibiotic-resistant pneumococci are serotypes included in PCV, the introduction of PCV has significantly reduced the burden of antibiotic-resistant pneumococcal infections.[28] In this study, performed 5 years after introduction of PCV10 in Ethiopia, the prevalence of penicillin-non-susceptible (PNSP) isolates was only 2.5% whereas 58% were intermediate. In a study on
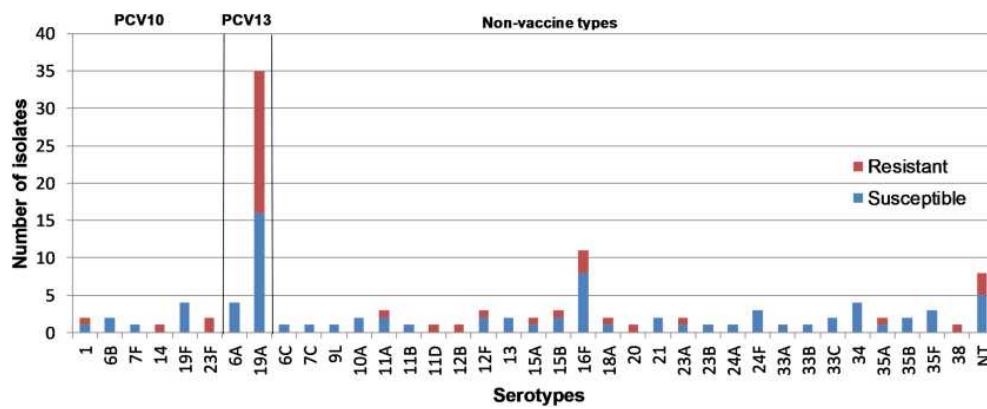
Figure 2 Pneumococcal serotypes from pediatric patients aged 0–15 years in Addis Ababa, Ethiopia and their susceptibility to erythromycin.

pneumococcal isolates from children with meningitis before the introduction of PCV10 in Ethiopia, 17% (8/46) were intermediate and there were no PNSP isolates reported. The isolates with intermediate penicillin MIC levels were serotype 19F (n = 4), 14 (n = 3), and 7 (n = 1).[29] In Kenya, a country which introduced PCV10 around the same time as Ethiopia, a similar trend of penicillin susceptibility has been reported. Penicillin susceptibility among pneumococcal isolates from children aged < 5 years indicated that prevalence of PNSP was low and remained unchanged in the pre-PCV10 period (2009), 2.4% and post-PCV10 period (2013), 2.7% while most isolates were intermediate (77.6% in 2009 and 74.8% in 2013).[30] In most countries that reported a decline in anti-microbial-resistant pneumococcal diseases, the prevalence of antimicrobial resistant vaccine-serotypes was high in the pre-PCV period.[11] In South Africa for example, 4 years after introduction of PCV7, incidence of invasive pneumococcal disease due to PNSP isolates among children aged < 2 years of age declined by more than 80%.[31] In Brazil, PCV10 was introduced in 2010 and analysis of penicillin non-susceptibility among pneumococcal isolates recovered from carriage and invasive disease indicated a decline in the occurrence of PNSP among IPD isolates but an increase among carriage strains. The differences in impact of PCV on the prevalence of PNSP serotypes in different countries and regions indicates that antimicrobial resistance is multifactorial and might be influenced by the different antibiotic use policies and prevalence and nature of serotypes that emerged after introduction of PCV.[11,32] This also reinforces the need for continued long-term surveillance of the dynamic changes in antimicrobial resistance among pneumococcal isolates due to PCV introduction.

In the current study, two out of the three PNSP isolates and most, 40.6% (28/69) of the penicillin intermediate isolates were serotype 19A and among serotype 19A isolates the majority (80%, 28/35) were intermediate. In Brazil, after introduction of PCV10, serotype 19A was the most important non-PCV10 serotype that emerged with increased prevalence in both carriage and disease.[32] In addition, the rate of PNSP isolates among serotype 19A isolates increased significantly (11.4% to 64%, $P < 0.001$).[33]

Reports from many parts of the world indicate that macrolide-resistant *S. pneumoniae* are now more common than PNSP.[34] In the current study, 33.6% of the pneumococcal isolates were resistant to erythromycin out of which half were serotype 19A. Our findings were similar to a study on antimicrobial resistance among nasopharyngeal pneumococcal isolates from pediatric outpatients in Northern Ethiopia (33.2%) performed after the introduction of PCV10.[35] In a study performed before the introduction of PCV10 in Ethiopia on the distribution of nasopharyngeal pneumococcal serotypes before and after mass azithromycin treatment for trachoma in Northern Ethiopia, the prevalence of pneumococcal serotype 19A was quite low (2%).[36] In the same study, the resistance level for azithromycin increased from 0% pre-treatment to 50% post-treatment as it did for most other serotypes, indicating the impact of treatment on selection of antibiotic resistant strains. In Brazil, prevalence of erythromycin resistance among serotype 19A isolates from children aged < 5 years of age increased from 20.5% pre-PCV10 period to 85.7% in 6–7 years after PCV10 introduction and factors such as the pressure of vaccine and antimicrobial use and introduction and spread of multidrug resistant clones have been cited as possible reasons for this increase.[33] In Ethiopia, although there is a lack of surveillance data on

antimicrobial resistance among different pneumococcal serotypes, the high prevalence of macrolide resistance especially among serotype 19A in this study may be due to the pressure of PCV10 and the use of antibiotics.

Results from a randomized double-blind trial that assessed the impact of PCV13 over PCV7 on reducing carriage of antibiotic-resistant pneumococci in Israel indicated that PCV13 significantly reduced the acquisition of pneumococci non-susceptible to penicillin, erythromycin, and clindamycin, mainly seen in serotypes 19F, 6A, and 19A.[37] It is also hoped that the introduction of PCV13 in Ethiopia will help to reduce acquisition of antibiotic-resistant pneumococci, especially serotype 19A.

In the current study, the most common macrolide resistance mechanism was macrolide efflux, identified in 55% of the macrolide resistant isolates. The results were similar to the only previous study in Ethiopia that identified macrolide resistance mechanisms.[17] In that study, performed before the introduction of PCV10 in Ethiopia, discrimination was not made between *mef*(A) and *mef*(E) genes. In the current study, macrolide efflux was the main macrolide resistance mechanism and *mef*(E) was the most common gene. Previous findings indicate that *mef*(E) is predominant in the United States, South Africa, and Asia while *mef*(A) is more common in Europe.[38] Although macrolide resistance encoded by *mef* class of genes in *S. pneumoniae* is thought to confer low- to mid-level resistance,[15] in our study, 65% of the strains with *mef*(E) had high level of resistance (MIC > 256 µg/mL). A recent study has indicated the ability of *S. pneumoniae* to generate high-level macrolide resistance by macrolide efflux and in the presence of macrolides, thus offering a fitness advantage to *S. pneumoniae*.[39]

In 40% (16/40) of the macrolide-resistant isolates, neither *erm*(B) nor *mef*(A/E) were detected. This indicates that other less common macrolide resistance mechanisms such as methyltransferase genes *erm*(A) and *erm*(C), the efflux pump gene *mef*(I), or mutations in 23S rRNA, L4, and L22 proteins might be responsible,[40] which requires further investigation.

We acknowledge that our study had some limitations. First, the number of isolates is relatively small and more isolates would have been useful. Second, the focus of our study was on the most common macrolide resistance mechanisms and we did not test the presence of the less common macrolide-resistance mechanisms. Despite these limitations, however, to the best of our knowledge, this is the first report of the distribution of penicillin- and macrolide-resistant pneumococcal serotypes and the prevalence of macrolide-resistant pneumococcal genotypes in the post-PCV10 era in Ethiopia.

Conclusions

Findings from the study indicate that penicillin resistance among *S. pneumoniae* isolates, 5 years after the introduction of PCV10 in Ethiopia, is low, with most PNSP isolates being intermediately susceptible. Resistance to macrolides on the other hand was high and half of the isolates of the most prevalent serotype 19A were resistant to macrolides. Among macrolide-resistant isolates, the most common mechanism of resistance was macrolide efflux, with predominance of *mef*(E). Continued studies on the impact of antibiotic treatment and PCV on the prevalence and distribution of antibiotic-resistant pneumococcal serotypes in Ethiopia are warranted and introduction of PCV13 would significantly enhance the coverage of the predominantly macrolide-resistant serotype 19A.

Ethics Approval and Consent to Participate

The study procedures were in accordance with the Helsinki Declaration. The study was approved by the AHRI/All Africa Leprosy Rehabilitation and Training Hospital (ALERT) Ethics Review Committee, Addis Ababa University Institutional Review Board, Yekatit 12 Medical College Ethics Committee and the National Research Ethics Review Committee (No. 310/194/2017). The parents and/or guardians of all participants gave written informed consent.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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