




Serotypes and Antimicrobial Susceptibility of Nasopharyngeal Isolates of *Streptococcus pneumoniae* from Children Less Than 5 Years Old in Egypt

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Amani El-Kholy¹
Magda Badawy²
Maha Gad¹
May Soliman¹

¹Department of Clinical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt; ²Department of Pediatrics, Faculty of Medicine, Cairo University, Cairo, Egypt

Purpose: *Streptococcus pneumoniae* (*S. pneumoniae*) is the etiology of severe and life-threatening infections in children less than 5 years old. Though pneumococcal conjugate vaccines (PCVs) are effective in the prevention of pneumococcal infections, yet they are not included in the National Immunization Program in Egypt pending the identification of pathogenic serotypes. As *S. pneumoniae* colonization of the pharynx predisposes to pneumonia and invasive pneumococcal disease (IPD) caused by the colonizing serotypes, identification of the nasopharyngeal (NP) serotypes can be a surrogate to the invasive serotypes. In this study, we aimed to 1. Identify the serotypes and antimicrobial susceptibility testing (AST) of *Streptococcus pneumoniae* colonizing the nasopharynx of Egyptian children younger than 5 years in two successive winter seasons. 2. Correlate the identified serotypes with vaccine coverage of the 13-valent conjugate pneumococcal vaccines (PCV13). 3. Compare the serotypes and AST of *S. pneumoniae* from NP to those of IPD that were routinely identified in our clinical laboratory during the study period.

Materials and Methods: The study was conducted in two successive winter seasons (December 2015–March 2016; December 2016–March 2017). We enrolled 334 children, aged 6 months to 5 years, attending the outpatient general clinics of Cairo University Children Hospital, excluding those with fever, signs of infection, history of antibiotic intake or hospitalization in the preceding month. We tested NP swabs for *S. pneumoniae* by culture and real-time PCR. Serotyping was performed by sequential multiplex PCR for all positive samples. AST was done to *S. pneumoniae* isolates by Vitek-2™ (BioMérieux, Marcy-L'Étoile, France). We included routinely detected *S. pneumoniae* from sterile body sites during the study period, and identified their serotypes and AST.

Results: PCR was positive for pneumococci in 217 out of 334 pharyngeal swabs (65%), including 186 typable samples. The most common serotypes were serotypes 1, 6ABC, 19 F, 5 and 18ABC. By culture, we isolated only 110 out of 334 pharyngeal swabs (32.9%). The theoretical coverage of the PCV13 vaccine for the detected serotypes was 77.4%. The AST of NP isolates revealed low susceptibility rates to all antimicrobials except for vancomycin, linezolid, levofloxacin and clindamycin. During the study period, we identified 40 IPD; 21 identified by PCR and 19 by culture. The commonest pneumococcal serotypes were 1, 18ABC, 6ABC and 5. The PCV13 coverage was 75%. By Vitek-2, the isolates showed 100%, 100%, 94.7%, 89.5%, 84.2%, 84.2% and 78.9% susceptibility to vancomycin, linezolid, clindamycin, levofloxacin, penicillin, cefotaxim and erythromycin, respectively.

Conclusion: Based on the serotype vaccine coverage and the emerging antimicrobial resistance of *S. pneumoniae*, PCVs will be valuable to Egyptian children.

Keywords: serotyping, *Streptococcus pneumoniae*, *S. pneumoniae*, antimicrobial resistance, penicillin non-susceptible, pneumococcal vaccines, immunization

Correspondence: May Soliman
Department of Clinical Pathology, Faculty of Medicine, Cairo University, 21 Gaber Ibn Hayan St, Dokki, Cairo, Egypt
Tel +20 2 1068800242
Email dr.maysheerif@gmail.com

Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is one of the most common pathogens causing a wide range of infections. Pneumococcal infections are prevalent in children, with adverse clinical and economic burden, particularly invasive pneumococcal diseases (IPD) and when infection is caused by resistant isolates.¹

Though the pneumococcal conjugate vaccines (PCVs) have proved to be effective, many middle-income countries are implementing PCVs at a slower rate than donor-funded low-income countries and wealthier developed countries.²

In Egypt, PCVs have not yet been included in the national children vaccination program as the availing data on pneumococcal serotypes are limited. In the winter season of 2013–2014, a study from Egypt detected nasopharyngeal (NP) carriage of *S. pneumoniae* in 56.5% of children less than 5 years; and the coverage of the PCV13 vaccine for the detected serotypes was 72.4%.³ However, as the NP pneumococcal serotypes show temporal variations in serotypes and antimicrobial resistance,⁴ more studies are deemed necessary. In this study, we aimed to:

1. Identify the serotypes (ST) and antimicrobial susceptibility testing (AST) of *S. pneumoniae* colonizing the nasopharynx of Egyptian children younger than 5 years in two successive winter seasons, and detect the temporal changes in serotypes in comparison to a recently published report.³
2. Correlate the identified serotypes with vaccine coverage of PCV13.
3. Compare the NP colonization of *S. pneumoniae* serotypes and AST to the serotypes and AST of *S. pneumoniae* routinely identified from IPD in our clinical laboratory during the study period.

Materials and Methods

Ethical approval was obtained from the Cairo University, Faculty of Medicine REC, that works in accordance with the Declaration of Helsinki under the number R-11-2018. Informed consent was obtained in Arabic language from the guardian.

Subjects

This study was conducted in two successive winter seasons (December 2015 –March 2016; December 2016–March 2017). We enrolled 334 children, aged 6 months to 5 years attending the outpatient general clinics of Cairo

University Children Hospital for non-infectious complaints. We excluded children with fever, signs of infection, history of antibiotic intake or hospitalization in the preceding month. The algorithm for laboratory testing is shown in Figure 1.

Methods

Collection of Nasopharyngeal (NP) Secretion

We collected NP secretion by flocked swabs (Copan Flock Technologies, Brescia-Italy) on STGG medium (skim milk, tryptone, glucose, and glycerol); swab shaft was cut with scissors and transported to the laboratory within 30 minutes according to WHO guidelines.⁵

Culture and AST

Each sample was vortexed for 10 seconds, and then 10 µL was streaked on a sheep blood agar plate (BAP). BAPs were incubated at 35°C in a CO₂ enriched atmosphere using a candle jar for 18 to 24 hours; the rest of the sample was aliquoted and stored at –80°C. Plates were examined and alpha-hemolytic colonies were tested for optochin susceptibility and bile-solubility.⁶ Optochin-susceptible bile-soluble isolates were identified as *S. pneumoniae* and tested for antimicrobial susceptibility using Vitek-2™ (BioMérieux, Marcy-L'Etoile, France) and interpreted following standard guidelines.⁷

Serotyping

DNA was extracted directly from specimens using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions. A pneumococcus-specific real-time PCR targeting the *lytA* and *Ply* genes was performed as described previously.³ *S. pneumoniae* serotyping was performed on RT-PCR positive samples, using the Centers for Disease Control and Prevention 8 *S. pneumoniae* serotyping procedure, African set.⁹ We conducted a sequential multiplex PCR on DNA extracted from biological samples. Forty-two primer pairs for serotypes 1, 2, 3, 4, 5, 6A/B/C, 7F/A, 7C/B, 8, 9V/A, 9N/F, 10A, 10F, 11A/D, 12A/F, 13, 14, 15A/F, 15B/C, 16F, 17F, 18A/B/C, 19A, 19F, 20, 21, 22A/F, 23A, 23B, 23F, 24A/B/F, 25F/38, 31, 33A/F, 34, 35A/C, 35B, 35F, 35A, 38 and 39 were grouped into eight multiplex reactions. *CpsA* (pneumococcal capsular polysaccharide synthesis gene) primers were included in each reaction mix as a confirmatory test. The PCRs were performed in 25 µL volumes, with the mixture containing multiplex PCR Master Mix (Qiagen, Valencia, CA) and primers (0.2–0.5

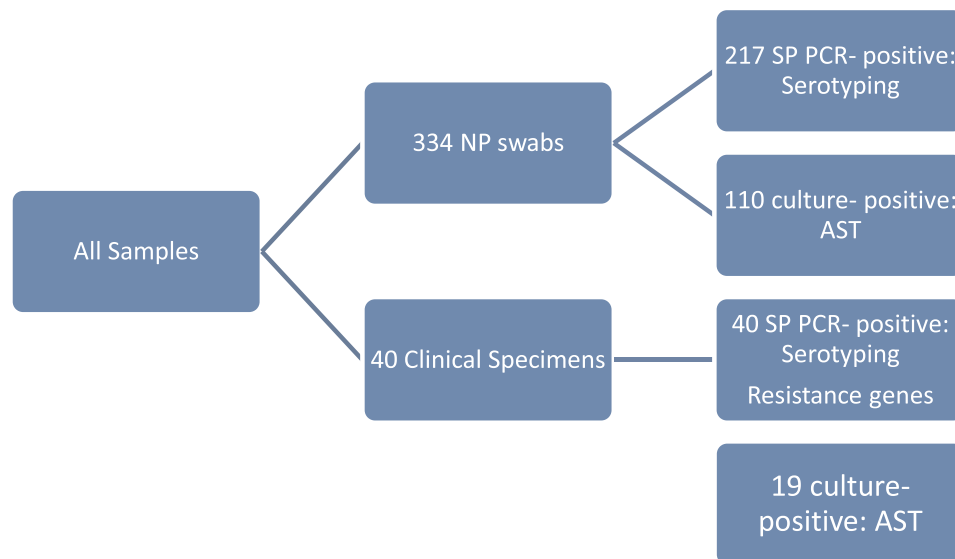


Figure 1 Algorithm for laboratory testing.

mM each); 5 ul DNA extract was used in each PCR. Amplification was performed in a Seeamp Thermal Cycler (Seegene) under the following conditions: 95°C for 15 min followed by 35 amplification cycles of 94°C for 30 s, 54°C for 90 s and 72°C for 60 s. A final hold was performed at 72°C for 10 min.

The PCR products were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer. Gels were stained with ethidium bromide (0.5 mg/mL), and gel images were recorded. The sizes of the PCR products were determined by comparison with the molecular size standard as well as with the bands of the respective controls.

Forty-one clinical isolates of pneumococci representing different serotypes and serogroups were used as a control set. The serotypes included 1, 2, 3, 4, 5, 6A, 6C, 7C, 7F, 8, 9N, 9V, 10A, 10F, 11A, 11B, 12F, 13, 14, 15A, 15C, 16F, 17F, 18C, 19A, 19F, 20, 21, 22F, 23A, 23B, 23F, 24F, 31, 33F, 34, 35B, 35F, 35A, 38, 39.

S. pneumoniae from IPD

During the study period, we detected 40 *S. pneumoniae* from sterile body sites (27 from blood, 5 from CSF and 8 from pleural fluid) out of 7200 clinical specimens routinely submitted to our laboratory affiliated to Cairo University Hospitals which provide a tertiary care service to patients coming from different governorates all over Egypt. Of those 19 were detected by culture and their AST was done by VITEK-2. The other 21 specimens were culture-negative and detected only by PCR; this could be attributed to the antibiotic intake before the collection of microbiological cultures,

especially before presenting to the hospital. For all 40 IPD samples, resistance to the penicillin and macrolides was studied by detection of the resistance genes PBP-2, *mef* and *erm* as follows.

Detection of Resistance Genes

Genotypic macrolide resistance (*ermB* and *mef*) and wild-type alleles of *pbp2b* typically associated with penicillin susceptibility were detected by CDC recommended multiplex real-time PCR protocol.¹⁰ Briefly, primers' sequences, concentrations and cycle conditions were selected according to Srinivasan et al.¹¹ Universal Taqman Master mix (Thermo Fischer, Foster City, CA) was used in a 25ul total reaction volume and operated on a 96 CFX Biorad real-time PCR machine.

Data Analysis

Data were statistically described in terms of mean \pm standard deviation (\pm SD), range, or frequencies (number of cases) and percentages when appropriate. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 17 for Microsoft Windows.

Results

Detection of *S. pneumoniae* from NP Samples

We enrolled 334 children, aged 6 months to 5 years, among the attending children to the outpatient general clinics of

Cairo University Children Hospital for non-infectious complaints. Male cases represented 118 (54.1%). By PCR, *S. pneumoniae* was detected in 217 (65%) most of them were isolated from male carriers who represent 54.1% of the children. Regarding the culture method, we isolated 110 *S. pneumoniae* out of 334 (32.9%).

Serotyping

The most frequently detected serotypes were serotypes 1, 6, 19F, 5, 18ABC, 14 and 19A (Table 1). The total vaccine coverage is 77.4% of typable *S. pneumoniae* (Table 1), considering the PCV13 valency as follows: 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F.

Antimicrobial Susceptibility

The AST revealed low susceptibility level to all antimicrobials except for vancomycin, linezolid, levofloxacin and clindamycin (100%100%, 90.9% and 72.7%, respectively) (Table 2).

Typing and AST of *S. pneumoniae* from IPD

Male patients represented 67.5% (27) out of 40 cases with IPD. Pediatric patients showed invasive pneumococcal

disease more than the adult (92.5% Vs 7.5%). The commonest serotypes were 1, 18ABC, 6ABC and 5 (Table 3). The IPD isolates showed 100%, 100%, 94.7%, 89.5%, 79%, 84.2% and 78.9% susceptibility to vancomycin, linezolid, clindamycin, levofloxacin, penicillin, cefotaxim and erythromycin, respectively. In the DNA of 40 invasive *S. pneumoniae*, PBP2b (wild type), *mef* and *erm* genes were detected at 27.5%, 75% and 42.5%, respectively (Tables 4 and 5). (Table 6) showed a comparison between antimicrobial susceptibility by vitek and the detected resistant genes by PCR for the 19 *S. pneumoniae* isolates from IPD. All *S. pneumoniae* isolates which were sensitive to penicillin by MIC expressed the wild type of PBP-2b and were detected by PCR except two isolates. On the other hand, the resistant isolates to erythromycin were positive for erythromycin-resistant genes (*mef2*, *erm*) except two isolates that showed false-positive erythromycin resistance genes.

Discussion

In this study, we identified the serotypes of *S. pneumoniae* NP carriage from healthy children or children with non-infectious diseases in two successive winter seasons. The

Table 1 Number, % and PCV13 Coverage of the Identified NP Serotypes

Serotypes	Number Typable 186	ST % per Typable SP	Serotypes	Number Typable 186	ST % per Typable SP
1	33	17.7	15B/15C	4	2.2
3	3	1.6	17F	4	2.2
4	4	2.2	18ABC	12	6.5
5	18	9.7	19A	8	4.3
6ABC	24	12.9	19F	20	10.8
7A/7F	3	1.6	21	1	0.5
7C/7B/40	1	0.5	22A/22F	2	1.1
8	2	1.1	23A	2	1.1
9V/9F	5	2.7	23F	4	2.2
9N/9L	2	1.1	25F	1	0.5
10A	2	1.1	33A/35F/37	1	0.5
11A/11D	3	1.6	34	8	4.3
12F/12A	5	2.7	35B	1	0.5
13	2	1.1	38	1	0.5
14	10	5.4			
Typable number	186		55.7%		
Number covered by PCV-13	144		77.4%		
non-typable per total samples	31		9.3%		
Number positive for SP	217		65.0%		
Number negative for SP	117		35.0%		
Total NP samples collected	334		100%		

Notes: Serotypes in boldface are those covered by 13-valent pneumococcal conjugate vaccine-13 (PCV-13).

Table 2 Antimicrobial Susceptibility of *S. pneumoniae* Colonizing the Nasopharynx

Antibiotic	Cultured Isolates (110)	
	Number of Sensitive Isolates	% Sensitivity
Ampicillin	36	32.7%
Vancomycin	110	100%
Linezolid	110	100%
Tetracycline	36	32.7%
Clindamycin	80	72.7%
Erythromycin	61	55.5%
Levofloxacin	100	90.9%
Sulfamethoxazole-trimethoprim	50	45.5%
Ceftriaxone	60	54.5%

most frequently detected serotypes from nasopharyngeal carriage were serotypes 1, 6, 19F, 5, 18ABC, 14 and 19A; and 77.4% of *S. pneumoniae* serotypes were covered by PCV-13.

The frequent carriage of ST 1, 6 and 19 F in the current study is in keeping with the recently published report from Egypt.³ In contrast, ST 5, 14 and 18 were not detected in the older study, while ST 34 that was frequent in the previous study was not detected in the current study. The 77.4% vaccine coverage of NP *S. pneumoniae* in this study is in agreement with the reported 72.4% coverage in the previous study.³

Surveillance of serotypes of *S. pneumoniae* is conducted in many countries. Our results are in broad agreement with studies on carriage in Egypt, Africa, the Middle East and other parts of the world.¹²⁻¹⁴

The temporal variation in serotypes occurs probably as a result of natural genetic transformation and bacterial interaction in the nasopharynx, which is rich in closely related microbial flora. This natural transformation has been known to play an important role in the evolution of the genomes of members of Genus *Streptococcus*.⁴ As an example, *Streptococcus mitis* group that are common NP colonizers together with *S. pneumoniae* has long been recognized as a reservoir of antibiotic resistance genes for *S. pneumoniae*, as well as a source for capsule polysaccharide variation, leading to resistance and vaccine escape.¹⁵

During the study period, we detected 19 *S. pneumoniae* isolates from IPD out of 7200 clinical specimens submitted to the laboratory. Additionally, we identified 21 *S. pneumoniae* by PCR from a sterile body site. The low number of IPD patients detected during the study period is

Table 3 Number, Percentage and PCV13 Coverage of Serotypes of 40 *S. pneumoniae* from IPD

Serotypes	Specimen	Number of Samples	%
1	Blood	8	20
3	CSF	1	2.5
5	Pleural fluid	3	7.5
6ABC	Blood	6	15
7A/7F	Blood CSF	2 1	7.5
7C/7B/40	Blood	1	2.5
9V/9F	CSF	1	2.5
11A/11D	Blood	1	2.5
12F/12A	Blood	1	2.5
13	Blood	1	2.5
14	Blood	1	2.5
15B/15C	Blood	1	2.5
17F	Blood	1	2.5
18ABC	pleural fluid	5	12.5
19A	Blood	1	2.5
19F	CSF	1	2.5
21	Blood	2	5
22F/22A	Blood	1	2.5
23A	Blood	1	2.5
Serotypes covered by PCV-13		30	75%

Note: Serotypes in boldface are those included in PCV-13. Vaccine coverage of IPD serotypes is 75%.

consistent with previous results from Egypt.¹⁶ In their hospital-based surveillance for laboratory-confirmed *S. pneumoniae* cases in children younger than 5 years, from 2008 to 2011, Draz et al isolated *S. pneumoniae* from 12 patients: four IPD and eight non-IPD out of more than 22,000 cultured specimen.¹⁶ The low detection rate may be attributed to antibiotic intake before the collection of microbiological cultures, especially before presenting to the hospital. Antibiotics are available over the counter in Egyptian pharmacies, and self-medication and purchasing without medical prescriptions are common practice in Egypt.^{17,18}

However, *S. pneumoniae* was more frequently identified from children with severe pneumonia in Egypt.^{16,19} In a study on 100 children with the severe community-acquired pneumonia admitted to ICU in Assiut (in southern Egypt), *S. pneumoniae* was isolated from 36% of the

Table 4 Antimicrobial Susceptibility of 19 *S. pneumoniae* Isolates from 19 IPD

	Pen	Amp	CTX	E	CD	lev	LZD	Van	Tet	SXT
Susceptible Number	15	15	16	15	18	17	19	19	13	14
%	79	79	84.2	78.9	94.7	89.5	100	100	68.4	73.7

cases. Compared to other patients, pneumococcal pneumonia patients presented with more severe clinical picture ($p < 0.001$) and resulted in higher mortality rate ($P = 0.45$).¹⁹ In contrast to our results, IPD is more frequently reported from other developing countries.²⁰

From pneumococcal IPD, the commonest serotypes were ST 1, 18ABC, 6 and 5; and the PCV13 coverage was 75% (Table 3). ST 1, 6, 18ABC and 5 were the commonest both in NP colonization and IPD, which proves the close relationship between colonization and IPD. NP colonization induces pro-inflammatory chemokines, along with damage to the respiratory epithelium caused by upper respiratory viral infection facilitates pneumococcal penetration of host tissues and progression to localized or invasive disease.²¹ Multiple studies were in accordance with ours regarding the serogroups detected in IPD; ST1 had a significantly higher invasive disease potential in children aged 0–23 and 0–59 months,²² serotypes 1 and 5 were likely to be about 3–4 times more invasive in children more than 2 years.²³ William et al declare that ST1 and 5 have commonly been viewed as “developing country” serogroups and cause 15–20% of IPD in Latin America, Asia, and Africa. In all these studies, the apparent differences in serotype distribution are influenced by age, population, geographic area, regional variations and time after PCV introduction.²⁴

Antimicrobial Susceptibility Testing (AST)

The nasopharyngeal isolates revealed low susceptibility to all antimicrobials except for vancomycin, linezolid, levofloxacin and clindamycin. Compared to results of Badawy

Table 5 Detection of Resistance Genes in DNA of 40 *S. pneumoniae* from IPD

	PBP2b	mef	erm
Number Positive	11	30	17
%	27.5	75	42.5

Abbreviations: SP, *Streptococcus pneumoniae*; ST, serotype; AST, antimicrobial susceptibility testing; NP, nasopharyngeal; MDR, multi-drug resistant; Pen, penicillin; Amp, ampicillin; CTX, cefotaxim; E, erythromycin; CD, clindamycin; LZD, linezolid; Van, vancomycin; Tet, tetracycline; SXT, sulfamethoxazole/trimethoprim; PCVs, pneumococcal conjugate vaccines; IPD, invasive pneumococcal disease.

et al,³ the new series showed an increase in the percentage of susceptibility to penicillin from 27.4% to 32.7%, clindamycin from 64.5% to 72.7%, erythromycin from 50% to 55.5% and sulfamethoxazole/trimethoprim from 3.3% to 45.5%. This might be due to the infrequent utilization of these groups as previously reported.²⁵ In contrast, susceptibility to ceftriaxone markedly decreased from 99.8% to 54.5%, which is consistent with increased utilization of third-generation cephalosporins in Egypt,²⁵ and reported resistance to third-generation cephalosporins.²⁶ Our rates of resistance are higher than a report from Lithuania that showed 56.7% of *S. pneumoniae* NP isolates were susceptible to all the antibiotics tested, and non-susceptibility to penicillin, erythromycin, clindamycin and trimethoprim/sulphamethoxazole was 15.8%, 21.3%, 16.9% and 27.3%, respectively.²⁷

However, invasive *S. pneumoniae* isolates showed lower rates of resistance compared to NP isolates. This is in accordance with a previous report that respiratory tract isolates showed more resistance to penicillin, TMP-SMX, cefuroxime and erythromycin, than invasive isolates, and were more likely to be multidrug resistant.²⁸ The Canadian Bacterial Surveillance Network also reported higher rates of penicillin NS for non-sterile compared with sterile isolates (16.6% versus 12.4%, $P = 0.005$), with respiratory tract specimens constituting 60% of the non-sterile isolates.²⁹ Similarly, in Quebec, noninvasive isolates demonstrated higher rates of resistance to macrolides, penicillin and cephalosporins.³⁰ The « Tracking Resistance in the United States Today (TRUST) » program in the United States reported higher rates of resistance among respiratory isolates versus blood for penicillin, azithromycin, TMP-SMX and levofloxacin ($P < 0.0001$ for all comparisons).³¹

Nevertheless, resistance genes were frequently detected in our invasive *S. pneumoniae* isolates. Molecular detection of resistance mechanisms has been proved to correlate with AST and to be helpful in rapid detection of resistance of pathogens causing life-threatening infections.³² Moreover, we could use this method in culture-negative IPD. Comparing conventional AST by VITEK-2 to the detection of resistance genes in 19 invasive *S. pneumoniae*, we found

Table 6 Comparison Between Antimicrobial Susceptibility by Vitek and the Detected Resistant Genes by PCR for the 19 Isolated *S. pneumoniae*

Molecular Detection of Resistant Genes	Vitek (MIC)			
	Penicillin		Erythromycin	
	(S)	(R)	(S)	(R)
Positive PBP-2b	15	2	-	-
Negative PBP-2b	2	0	-	-
Negative Erythromycin Gene	-	-	15	0
Positive Erythromycin Gene	-	-	2	2

good agreement (78.9% and 89.5% for penicillin and erythromycin genes, respectively). However we encountered disagreement regarding penicillin resistance in 4 isolates; 2 susceptible isolates with negative wild type PBP-2b and 2 resistant isolates with positive wild-type PBP-2b. Penicillin resistant isolates giving positive results for the wild type PBP2b, might be explained by amino acid substitutions near the sequence encoding the downstream KTG motif which can confer PEN resistance¹¹ or other mutations in different genes such as PBP2x and PBP1a.³³ On the other hand, penicillin-sensitive isolates giving negative result for the wild-type PBP2b need to be further studied and sequenced to uncover the unique genetic resistance profile of the Egyptian pneumococcal isolates that might have different substitutions at other sites. Regarding erythromycin, two susceptible isolates had false-positive *mef-A* and *erm-B* genes. This false positivity indicates the presence of similar resistance genes from other microorganisms in the clinical specimens, leading to cross-reactivity. Additionally, the mosaic genes that encode low-affinity PBP genes are products of recombination involving horizontal transfer from closely related species. Similarly, *erm(B)* and *mef(A)* macrolide-resistant genes in *S. pneumoniae* are highly homologous to genes in other *Streptococcus* species.³⁴ On the contrary, failure to detect the resistance genes (false negative) might be potentially conferred through other previously described mechanisms such as mutations in ribosomal genes encoding 23S rRNA and riboproteins L4 and L22.³⁵

Conclusion

The results of this study confirm the high carriage rate of *S. pneumoniae* among infants and children, and the substantial increase in emerging MDR strains in Egypt. As the PCV provides very good coverage to the *S. pneumoniae* serotypes causing colonization and IPD in Egypt, and considering the vulnerability of the younger age group

and high-risk population, we recommend including pneumococcal conjugate vaccines in the national vaccination program in Egypt.

In response to the high toll of pneumococcal infections, the global community needs to recognize the impediments to vaccine introduction into Low Middle-Income Countries including Egypt. Improving PCV access could help decrease the incidence of pneumococcal infections and reduce the selection pressure for pneumococcal antimicrobial resistance.²

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Disclosure

The authors report no conflicts of interest in this work.

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