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ORIGINAL ARTICLE

Serotype distribution and antibiotic resistance of *Streptococcus pneumoniae* isolates collected from unvaccinated children with pneumonia at a province in central Vietnam

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ABSTRACT

Background and Objectives: Identification of pnemococcal serotypes and antimicrobial resistance provides helpful information for the use of suitable vaccines and antibiotics; however, very limited data is available on these issues in Vietnam. The present study aimed to find the serotype distribution and drug resistance patterns of *Streptococcus pneumoniae* isolated from unvaccinated children less than 5 years of age with pneumonia at a province in centre Vietnam.

Materials and Methods: A total of 126 clinical pnemococcal strains isolated from unvaccinated children less than 5 years of age with pneumonia at the Nghe An province, Vietnam between Nov 2019 and Mar 2021. All strains were identified using conventional microbiological method, VITEK® 2 Compact system, specific PCR and sequencing. The serotypes and antimicrobial resistance patterns of pnemococcal strains were determined using the multiplex PCR assays and VITEK® 2 Compact system.

Results: The results showed that, eight different pneumococcal serotypes were identified. The most common serotypes were 19F (67.46%), followed by 23F (10.32%), 19A (9.52%), 6A/B (3.17%), 15A (2.38%), 9V (3.17%), 11A (1.59%) and 14 (0.80%), respectively. More than half of the pneumococcal strains were non-susceptible to penicillin. The resistance rate to ceftriaxone and cefotaxime were 41.3% and 50.8%. The percentage of pneumococci strains resistant to clarithromycin, azithromycin, erythromycin, cotrimoxazole, tetracyclin, and clindamycin were more than 93% of all strains. All pneumococcal serotypes were highly resistant to clarithromycin, azithromycin, erythromycin, cotrimoxazole, and clindamycin.

Conclusion: Our findings showed high antibiotic resistance rates of the strains causing pneumococcal pneumonia, mostly macrolide resistance, among unvaccinated children.

Keywords: *Streptococcus pneumoniae*; Serotypes; Antibiotic resistance; Children; Pneumonia

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INTRODUCTION

The bacterium *Streptococcus pneumoniae (S. pneumoniae*, pneumococcus) causes pneumococcal disease. This is a Gram-positive, facultative anaerobic bacterium that is an important pathogen causing community pneumonia, sinusitis, otitis media, and invasive infections such as bacteremia and bacterial meningitis, which are the leading causes of morbidity and mortality among children less than 5 years of age (1-3). The occurrence of diseases caused by this etiological agent are a prominent global public health issues (4, 5). According to the study reported by Wahl et al. (2018), diseases caused by *S. pneumoniae* kill 317,300 children under five years of age every year, mostly in lower income countries (6). *S. pneumoniae* also occurs as a cause of invasive infections in elderly persons (7).

Pneumococcus is currently divided into more than ninety serotypes based on the antigenic capsular polysaccharide (CPS) (8). The CPS is an important virulence factor of *S. pneumoniae* and of the pneumococcal serotypes, serotypes 6, containing four confirmed serotypes (6A-6D), are reported to account for the most common serotypes of pneumococcal disease worldwide (5, 9, 10). Epidemiological studies around the world have showed that distribution of pneumococcal serotypes varies by age and geographical area (11-13). According to previous studies, although there are many pneumococcal serotypes, only certain types lead to invasive diseases worldwide such as serotypes 1, 4, 6A/6B, 7F, 9V, 14, 15B/15C, 18C, 19F, 19A, and 23F, which were found to account for about 80-90% of invasive pneumococcal disease (IPD) in children, especially in unvaccinated areas (2, 14, 15). Therefore, the type of pneumococcal vaccines should be selected in accordance with the circulating serotypes in each country (5, 11).

The antibiotic treatment for pneumococcal infections seems to be the primary choice. However, increasing antibiotic drug-resistance of pneumococcus, mainly to β-lactams, has been noted worldwide, especially in Asia, that has made the role of antibiotics is limited (11, 13). This showed that the importance of disease prevention (11). Vaccines have demonstrated to be an effective means of preventing pneumococcal disease worldwide (13). In the US, after the introduction of seven-valent pneumococcal conjugate vaccines (PCV7) to prevent pneumococcal diseases, the rate of IPD has significantly reduced from >200 cases/100 000 persons to >50 cases/100 000 persons (7). To date, 146 member countries out of WHO members have added pneumococcal conjugate vaccine (PCV) into their National Immunization Program (16). Nevertheless, only about 55% (approximately 74 million) of the global infant population are receiving PCV (7).

In addition, antibiotic resistance in *S. pneumoniae* strains is rising in all parts of the world, including Vietnam (17). Thus, an understanding of the serotype distribution and antibiotic resistance patterns of pneumococcus is necessary to guidance for the use of suitable vaccines and antibiotics (3). The aim of this study was to determine the serotypes and patterns of antibiotic resistance of *S. pneumoniae* isolated from unvaccinated children less than 5 years of age with pneumonia at a province in centre Vietnam.

MATERIALS AND METHODS

Bacterial isolates and identification of *S. pneumoniae*. In current study, a total of 126 *S. pneumoniae* clinical isolates were isolated from sputum samples of pneumonia children, aged between 2 and 59 months, at the Nghe An Obstetrics and Pediatrics Hospital (500 beds), Nghe An province, Vietnam, during the period between November 2019 and March 2021. Sputum specimens for each patient were taken by trained nurses using a clean suction and were then transported to the clinical microbiology laboratory within 2 h for isolation of *S. pneumoniae*. All samples were inoculated onto agar plates containing 5% sheep blood (Himedia, India) at 37°C in 5% CO_2 atmosphere for 18-24 h. The samples that no growth on the agar after 24 h were followed up for a further 24 h before being pronounced as negative. Colonies of suspected isolates was taken to identify as *S. pneumonia* using conventional microbiological method (Gram staining, the alpha hemolysis test, the optochin sensitivity test) in combination with the VITEK® 2 Compact system (bioMérieux, North Carolina 27712, USA) according to the manufacturer's instructions and PCR analyses using species-specific primers as described previously (1). All isolates were stored at -80°C in cryotubes containing trypticase soy broth (Merck, Germany), 20% glycerol (Merck, Germany) and 10% horse serum for further analysis.

Determination of pneumococcal serotypes. Ge-

nomic DNA of *S. pneumoniae* was extracted from the bacterial cultures using G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea), following the manufacturer's protocol. First of all, the pneumococcal isolates were confirmed by molecular method using the specific primer pair of *cpsA*-F (5'-GCA GTA CAG CAG TTT GTT GGA CTG ACC-3') and *cpsA*-R (5'-GAA TAT TTT CAT TAT CAG TCC CAG TC-3') (Integrated DNA Technologies, USA) for amplification of *cpsA* gene (8). And then, the most common serotypes of *S. pneumoniae* isolates were identified by multiplex PCR (mPCR) using twenty-one capsular specific primer pairs (Table 1) as described in previous reports (1, 8). The capsular types were collected on five groups as follows: Types 14, 19A, 19F and 23F; 6A/B, 9V, 15A and 15B/C; 1, 3, 10A and 11A; 4, 5, 7C and 17F; 7F, 8, 12A, 20 and 23B (Table 1). The mPCR reactions were carried out in 25 µl volumes containing 2 µl of DNA solution, 12.5 µl 2× Master mix (Cat.# M7505, Promega, USA), 0.5 µl of each primer (0.2 µM) and distilled water up to 25 µl. Thermal cycling was performed in Thermo Mastercycler Gradient system (Thermo Fisher Scientific, USA) under the following conditions: 94°C for 5 minutes; followed by 35 cycles at 94°C for 45 seconds, 54°C for 45 seconds and 65°C for 150 seconds; and a final extension of 72°C for 10 minutes. The mPCR products were analyzed on a 2% agarose gel containing 0.5 µg/ml ethidium bromide at 100V for 60 minutes and visualized with UV transillumination (UVP, Canada). The sizes of the mPCR products were determined by 100bp DNA Ladders (Cleaver, UK). Pneumococcal isolates that could not be serotyped by mPCR were classified as non-typeable. Total DNA isolated from *S. pneumoniae* strain ATCC 49619 were used for quality control.

16S rRNA gene sequencing. Two PCR primers, namely 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'), were chosen to amplify the 16S rDNA gene (18). PCR products of 16S ribosomal RNA genes from twenty-two isolates were randomly selected and were sent to Apical Scientific Sdn. Bhd (Selangor, Malaysia) for purification and automatic DNA sequencing, using the same primer pair. Species confirmed *S. pneumoniae* isolates were accurately examined by two-directional sequencing. The 16S rRNA gene sequences of these strains were deposited in the DDBJ/EMBL/GenBank databases under accession number MW672550-MW672562 and MZ007491-MZ007499, respectively.

Antimicrobial susceptibility testing. The antimicrobial susceptibility tests of each isolate with penicillin (PEN, 0.0625-8.0 µg/mL), cefotaxim (CXM, 0.125-8.0 µg/mL), ceftriaxone (CEF, 0.125-8 µg/mL), chloramphenicol (CLP, 1.0-16.0 µg/mL), azithromycin (AZM, 0.125-8.0 µg/mL), clarithromycin (CLA, 0.25-16.0 µg/mL), erythromycin (ERY, 0.125-8.0 µg/mL), clindamycin (CLI, 0.25-1.0 µg/mL), levofloxacin (LEV, 0.25-16.0 µg/mL), linezolid (LIN, 2.0-8.0 µg/mL), moxifloxacin (MXF, 0.0625-4.0 µg/mL), rifampicin (RIF, 0.0625-4.0 µg/mL), tetracyclin (TET, 0.25-16.0 µg/mL), vancomycin (VAN, 0.125-8.0 µg/mL) and trimethoprim-sulfamethoxazole (SXT, 10.0-320.0 µg/mL) were performed for each strain using VITEK® 2 Compact system according the manufacturer's instructions. The breakpoints used for *S. pneumoniae* were classified in accordance with the Clinical and Laboratory Standards Institute (CLSI) 2020 criteria. For quality control of the susceptibility tests, *S. pneumoniae* ATCC 49619 was chosen as the reference strain.

Statistical analysis. The statistical analysis was carried out by IBM SPSS Statistics software, version 20.0 developed by IBM Corp. (Armonk, NY, USA). Chi-squared and Fisher's exact tests were performed to check the significance of the data. P values less than 0.05 were considered significant statistically. The 16S gene sequences of pneumococcal isolates were compared to the publicly available DNA sequences in the Genbank databases, using BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Ethics approval and consent to participate. The purpose and benefits of the current study were informed to parents/legal guardians of each participant who also signed a written informed consent before the study procedure was performed. The study protocols was accepted by the Ethical Committee of the National Institute of Malariology, Parasitology and Entomology (Ha Noi, Vietnam) in March 2018 (ethics code: 225/QĐ-VSR). Furthermore, this study is based on the Declaration of Helsinki Principles.

RESULTS

All of the 126 individual isolates which were *S.*

Table 1. Primers used to confirm and identify serotypes of *S. pneumoniae*

Reaction	Serotype/ Primer	Primer sequence (5'-3')	Product size (bp)
1	14-F	CTT GGC GCA GGT GTC AGA ATT CCC TCT AC	208
	14-R	GCC AAA ATA CTG ACA AAG CTA GAA TAT AGC C	
	19A-F	GTT AGT CCT GTT TTA GAT TTA TTT GGT GAT GT	478
	19A-R	GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG	
	19F-F	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	304
	19F-R	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG	
	23F-F	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	384
	23F-R	CAC AAC ACC TAA CAC ACG ATG GCT ATA TGA TTC	
2	6A/B-F	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	250
	6A/B-R	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	
	9V-F	CTT CGT TAG TTA AAA TTC TAA ATT TTT CTA A	753
	9V-R	GTC CCA ATA CCA GTC CTT GCA ACA CAA G	
	15A-F	ATT AGT ACA GCT GCT GGA ATA TCT CTT C	436
	15A-R	GAT CTA GTG AAC GTA CTA TTC CAA AC	
	15B/C-F	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA	496
	15B/C-R	CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C	
3	1-F	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	280
	1-R	CCAAAGAAAATACTAACATTA TCA CAA TAT TGG C	
	3-F	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	371
	3-R	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	
	10A-F	GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC	628
	10A-R	GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C	
	11A-F	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	463
	11A-R	GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC	
4	4-F	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	430
	4-R	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G	
	5-F	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG	362
	5-R	GCTCGATAAACATAATCAATATTTGAAAAA GTA TG	
	7C-R	CTATCTCAGTCATCTATTGTTAAAGTTTACGACGGGA	260
	7C-R	GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC	
	17F-F	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	693
	17F-R	GAT GTA ACA AAT TTG TAG CGA CTA AGG TCT GC	
5	7F-F	CCT ACG GGA GGA TAT AAA ATT ATT TTT GAG	826
	7F-R	CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC	
	8-F	GAT GCC ATG AAT CAA GCA GTG GCT ATA AAT C	294
	8-R	ATC CTC GTG TAT AAT TTC AGG TAT GCC ACC	
	12A-F	ACT CTT CCA AAT TCT TAT GCT TTT ATT GAT TC	656
	12A-R	ATG AAT GAG AAA AGG AAC TTA AAA TTC ATA GC	
	20-F	GAG CAA GAG TTT TTC ACC TGA CAG CGA GAA G	514
	20-R	CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC	
	23B-F	TTG TTA GTG GTA TTA AAT TGG GGA CTA CTA GG	216
	23B-R	ATA CCT ATC TGA AGT GTT ATT AAC CCA CCA AC	
Positive control	*cps*A-F	GCA GTA CAG CAG TTT GTT GGA CTG ACC	160
	*cps*A-R	GAA TAT TTT CAT TAT CAG TCC CAG TC	

pneumoniae culture-positive also indicated a positive PCR result for the *cpsA* gene (Fig. 1). 22 sequences of the 16S rDNA regions of different pneumococcal isolates were also deposited in the NCBI database under accession number MW672550-MW672562 and MZ007491-MZ007499, respectively.

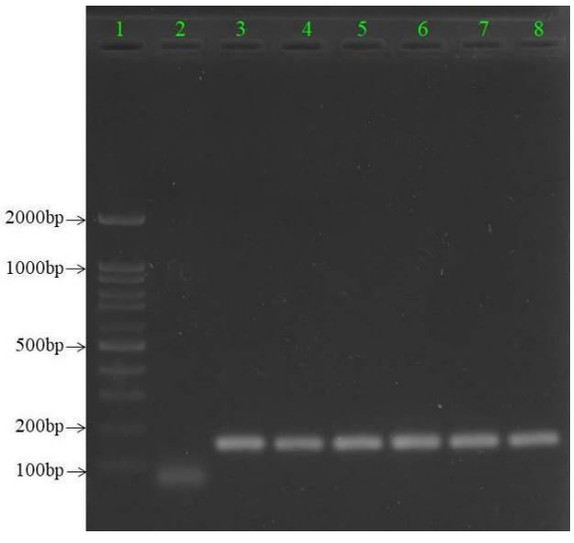

Fig. 1. Gel electrophoresis of *S. pneumoniae*-specific PCR products targeting the 160 bp *cpsA* gene
Lane 1: DNA Ladder 100 bp Standard; lane 2: negative control; lanes 3-7 (strain Sp8107, Sp8279, Sp8281, Sp8294, and Sp8298): clinical samples; lane 8: positive control

By multiplex PCR assays, of the 126 *S. pneumoniae* isolates analyzed, 124 strains (98.41%) could be serotyped, of which the eight different pneumococcal serotypes were classified. Only one serotype per patient was detected. The remaining 2 isolates (1.59%) could not be serotyped. The serotype distribution is shown in Figs 2 and 3.

Pneumococcal serotype distribution varied between age groups (Fig. 4), but the difference was not statistically significant between the two groups ($p > 0.05$).

Table 2 showed the trends of antimicrobial resistance patterns of *S. pneumoniae* strains. Accordingly, the observed resistance rates of 126 *S. pneumoniae* isolates to CLA, AZM, SXT, TET, CLI, ERY, CEF, and CXM were high, i.e. 100% (126), 100% (126), 93.7% (118), 96% (121), 96% (121), 99.2% (125), 41.3% (52), and 50,8 (64), respectively. This bacteria showed 100% susceptibility to the RIF, CLP, VAN, LIN and MXF. The susceptibility rates of pneumococcus to levofloxacin were 97.6%.

Antimicrobial resistance of pneumococcus among

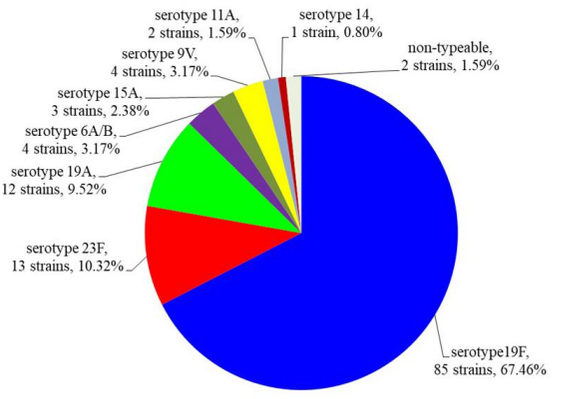

Fig. 2. The distribution of the pneumococcal serotypes

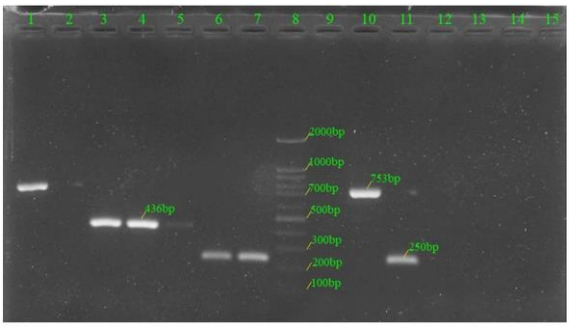

Fig. 3. The multiplex PCR patterns of serotypes 6A/B, 9V, 15A and 15B/C (reaction 2)
Lanes 1 and 10 denoted to those of serotype 9V; lanes 3-5 denoted to those of serotype 15A; lanes 6, 7 and 11 denoted to those of serotype 6A/B; lanes 2, 9, 12-14 denoted to those of non-typeable; lane 8: DNA Ladder 100bp Standard; lane 15: negative control.

different serotypes is shown in the Table 2. These results indicated that the observed resistance rates of serotype 19F were highest. All of 8 serotypes exhibited high rates of resistance to macrolides, tetracyclin and clindamycin.

DISCUSSION

Invasive pneumococcal disease is known to be a major cause of morbidity and mortality among children under five years of age, especially those under 2 years of age, although preventive actions have been implemented in many countries (1, 19). According to the previous studies, the distribution of pneumococcal serotypes, which plays an important role in

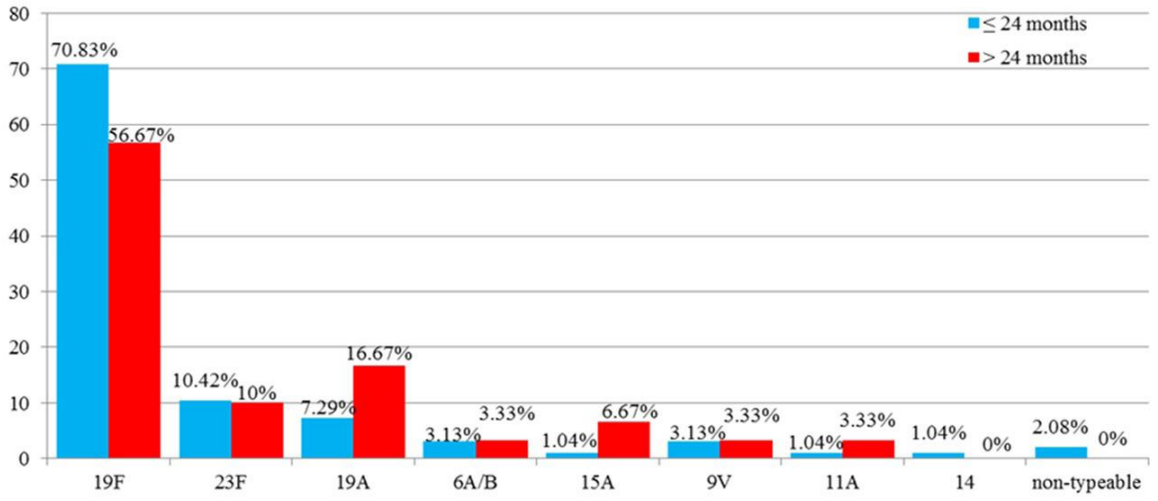

Fig. 4. Serotype distribution according to age groups

Table 2. Antimicrobial resistance rates of 126 pneumococcal isolates of different serotypes against 16 antimicrobial agents

Antibiotic	% of isolates			Resistance of serotypes (%)								
	S	*I*	*R*	19F	23F	19A	6A/B	15A	9V	11A	14	NT
				No. 85	No. 13	No. 12	No. 4	No. 3	No. 4	No. 2	No. 1	No. 2
PEN	46.8	48.4	4.8	4.7	0	8.33	**0**	33.33	0	0	0	0
CXM	22.2	27.0	50.8	64.7	7,7	25	50	66.7	25	0	0	0
CEF	25.4	33.3	41.3	55.3	7.7	0	50	0	25	0	100	0
LEV	97.6	0	2.4	3.5	0	0	0	0	0	0	0	0
MXF	100	0	0	0	0	0	0	0	0	0	0	0
ERY	0.8	0	99.2	100	100	100	100	100	100	50	100	100
CLI	4.0	0	96.0	95.3	100	100	100	100	100	50	100	100
LIN	100	0	0	0	0	0	0	0	0	0	0	0
VAN	100	0	0	0	0	0	0	0	0	0	0	0
TET	4.0	0	96.0	96.5	100	83.3	100	100	100	100	100	100
CLP	100	0	0	1.2	0	0	25	0	0	0	0	0
RIF	100	0	0	0	0	0	0	0	0	0	0	0
SXT	5.5	0.8	93.7	98.8	92.3	100	100	0	75	50	100	50
AZM	0	0	100	100	100	100	100	100	100	100	100	100
CLA	0	0	100	100	100	100	100	100	100	100	100	100

Abbreviation: S susceptible, I Intermediate, R Resistant; NT non-typeable

the cause of invasive infections, varies in different geographical area (11-13). Thus, routine screening for local serotype distribution of *S. pneumoniae* was necessary to inform the developing a safe and effective vaccine and guidance for the use of appropriate antibiotics (3). According to the WHO, universal vaccination is the best way against pneumococcal disease (2).

The epidemiological data from around the world have indicated that serotypes 1, 4, 6A/B, 7F, 9V, 14, 15B/C, 18C, 19F, 19A, and 23F represent around 80–90% of IPD in children, especially in unvaccinated areas (2, 14, 15). The current study detected pneumococcal serotypes 6A/B, 9V, 11A, 14, 15A, 19F, 19A, and 23F among unvaccinated children under five years of age in Nghe An province. The three major

serotypes 19F, 19A, and 23, were found to account for about 90% of *S. pneumoniae* isolates, while serotypes 6A/B, 9V, 11A, 14, and 15A were represented in lower percentages, ranging from 0.80 to 3.17%. Notably, serotype 19A has a rather high prevalence. The serotype distribution in this study was quite similar to those found previous studies in southern Vietnam, ASEAN countries, and Taiwan (20-24). Serotypes 6B, 23F and 19F are the most prevalent among children in Japan, while serotypes 1, 5, 6ABC, and 19F predominate in Egypt (25, 26). Serotype 14 was most common among strains from Paulo, Brazil and Casablance, Morocco (27, 28). Serotypes 23F, 14 and 3 are the most common in Tehran, Iran (11). In China, several studies have demonstrated the distribution of serotypes of *S. pneumoniae* varies between cities and different years (3, 29, 30). The results from different studies indicate that the prevalence of pneumococcal serotypes varies different depending on the population, region, and change over time (29, 31, 32). Thus, additional investigations should be carry out to identify the pneumococcal serotypes in different regions of Vietnam to provide information for the development of appropriate vaccines.

The emergence of resistance to antibiotics in pneumococci is increasing and it is becoming increasingly important predictive factor since it is directly related to persistent disease or disease mortality (3, 33, 34). The results of this study indicated that antimicrobial resistance patterns of *S. pneumoniae* is a matter of great concern. The resistance rates of *S. pneumoniae* isolates to AZM, CLA, CLI, ERY, SXT, and TET were higher than 96%. The previous studies in China, Taiwan and other Asian countries, including Vietnam also indicated bad *in vitro* activity of macrolides (ERY, CLA and AZM), lincosamide (CLI), tetracyclines (TET), and SXT against *S. pneumoniae* isolates (3, 23, 24). Our results suggested that these antibiotics are not appropriate for the treatment of pneumococcal disease in Vietnam. Besides, the rates of decreased susceptibility to penicillins and cephalosporins showed a rising trend. This result is in agreement with previous studies conducted in China, Taiwan and Vietnam, where more than 50% of patients infected with non-susceptible to penicillins and cephalosporins (3, 24, 29, 30, 35). In our study, all the *S. pneumoniae* strains were susceptible to RIF, CLP, VAN, MXF and LIN. Our findings also indicated the prevalence of LEV resistance in *S. pneumoniae* isolates were low (2,4%). The results of current study have shown that RIF, CLP, VAN, MXF, LIN and LEV may provide an opportunity for treating β-lactam, macrolides, lincosamide, tetracyclines, and cotrimoxazole-resistant pneumococcal disease in Vietnam.

In the present study, the prevalence of multidrug resistance of all eight serotypes were 100%. This rate was higher in the current research than previously findings in southern Vietnam (20-22). Notably, the high rates of antimicrobial resistance of serotype 19A were observed. This serotype is not covered by the PCV-7, thus the use of these vaccines may not be effective in preventing pneumococcal disease (3, 11). Therefore, PCV-13 should be recommended for future vaccination in Nghe An because of its broader serotype coverage.

CONCLUSION

In the current study, eight different pneumococcal serotypes were identified in Nghe An, Vietnam. Among that, 19F, 23F and 19A were the most prevalent serotypes. The high frequency of serotype 19A was a notable characteristic. In addition, the rate of antibiotic resistance of *S. pneumoniae* is considerable. Cautious use of antibiotics is extremely important and necessary to prevent the appearance of resistant pneumococci.

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