

Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* among children with acute otitis media in Marrakech, Morocco

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ABSTRACT

Background and Objectives: *Streptococcus pneumoniae* (*S. pneumoniae*) is one of the most frequent pathogens leading to a variety of clinical manifestations. The effects of *S. pneumoniae* carriage on acute otitis media (AOM) are poorly studied. The study aimed to assess the serotype's distribution and antimicrobial susceptibility in children with AOM after the implementation of the pneumococcal conjugate vaccine (PCV) in Morocco.

Materials and Methods: We conducted a prospective study of AOM children aged 6 to 36 months who visited pediatric centers in Marrakesh between January to June 2018. Parents were asked to complete a questionnaire and a swab was collected from each child. The *S. pneumoniae* strains were further identified (Hemolysis, optochin sensitivity, and agglutination test), serogrouped (IMMULEX PNEUMOTEST agglutination test), serotyped (Real time PCR) and tested for antimicrobial susceptibility.

Results: The *S. pneumoniae* carriage rate was 49.7% (87/175). As estimated, non-vaccine serotypes (NVT) were most prevalent (51/63; 81%). The most frequent serotypes were 6C/6D (12.7%), 10 (9.5%), and 19B/19C (9.5%). The *S. pneumoniae* strains that were isolated showed a diminished susceptibility to penicillin G with a rate of 27.5%. Penicillin non-susceptible pneumococci (PNSP) was mostly associated with NVT. More than 90% of *S. pneumoniae* isolates were susceptible to chloramphenicol (97.5%), clindamycin (97.5%), erythromycin (97.5%), levofloxacin (97.5%), pristinamycin (97.5%), gentamicin (92.5%), and teicoplanin (92.5%).

Conclusion: Important nasopharyngeal carriage prevalence was reported among children with AOM. The study showed that new NVT are emerging, including 6C/6D and 10. Furthermore, susceptibility was significantly higher against all antibiotics tested except for penicillin G and amoxicillin.

Keywords: Otitis media; Preschool children; Colonization; *Streptococcus pneumoniae*; 10 valent pneumococcal vaccine; Serotyping; Antibacterial drug resistance

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INTRODUCTION

Acute otitis media (AOM) continues to be among the most prevalent infectious diseases in early childhood worldwide (1). It is an inflammation of the middle ear characterized by a viscous effusion, fever, otalgia, otorrhea, and conjunctivitis (2, 3). Thus, AOM occurs most often in children aged less than 5 years and is a primary reason for pediatric consultations and antibiotic prescriptions (4). Predominant bacteria that cause AOM are *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* (5). However, it is known that *S. pneumoniae* is the main cause of AOM in approximately half of the global cases (6-8).

Pneumococcal infections are usually preceded by asymptomatic colonization of the nasopharynx (9). According to the diversity of capsular types, *S. pneumoniae* is divided into 101 distinct serotypes, where only serotypes 6A, 6B, 14, 19A, 19F, and 23F are frequently associated with AOM (10-12). Indeed, pneumococcal vaccination remains an effective means against the pneumococcal serotypes causing AOM (13-16). The pneumococcal conjugate vaccines (PCV) have dramatically reduced the proportion of AOM episodes caused by *S. pneumoniae* in young children (17).

In Morocco, PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) was introduced in the national immunization program (NIP) during 2010 and was later replaced by PCV10 (1, 4, 5, 6B, 7F, 9V, 14, 19F, 18C, and 23F) in July 2012. Following the implementation of these vaccines, no survey providing reliable epidemiological information on nasopharyngeal colonization among children suffering from AOM has been conducted. This study aimed to determine the colonization rate, the distribution of serotypes covered by PCV10, and the antibiotic resistance of *S. pneumoniae*, among AOM children, after PCV introduction.

MATERIALS AND METHODS

Study type and population. This prospective study was conducted from January to June 2018. Children who were aged 6-36 months and vaccinated against *S. pneumoniae* as well as admitted as outpatients by private pediatric centers in Marrakesh because of AOM were enrolled in this study. Each case

was defined based on the presence of the following symptoms and signs: earache and fever during a common cold illness combined with otoscopic confirmation of bulging, opacification with congestion, or a perforated tympanic membrane. Children were excluded if they had taken antibiotics one week prior to the enrollment. A questionnaire containing demographic (age, gender, and type of childcare) and clinical (fever, conjunctivitis, earache, and otorrhea) information was completed for each patient. The child's immunization history was checked and recorded.

Specimen collection and processing. Nasopharyngeal specimens were collected using a sterile swab containing transport medium and transferred within 2h at 4-8°C to the Bacteriology-Virology Laboratory of the Avicenna military Hospital in Marrakesh. Each swab was streaked on Colistin-Nalidixic Acid Agar supplemented with 5% of blood. The plates were incubated overnight at 37°C in a 5% CO₂ atmosphere.

Identification of *S. pneumoniae*. The pneumococcus isolates were preliminarily determined based on morphology, optochin susceptibility, and agglutination test (Slidexpneumo-Kit Bio Mérieux) (18, 19). Every single alpha-hemolytic colony was selected from the primary culture and a secondary culture was prepared to obtain pure growth.

Serogrouping / serotyping. The detection of serogroup/type was first performed using the IMMULEX PNEUMOTEST agglutination test (Staten Serum Institut Copenhagen, Denmark) and secondly using the real-time polymerase chain reaction (RT-PCR) in conformity with the protocol and guidelines published by the Centers for Disease Control and Prevention (CDC). Isolates that showed no agglutination were classified as non-typeable.

Antimicrobial susceptibility testing. The antimicrobial sensitivity testing was performed using the disk diffusion method on Mueller Hinton agar (MHA) supplemented with 5% of sheep blood. The antimicrobials tested were tetracycline, erythromycin, clindamycin, gentamicin, levofloxacin, teicoplanin, vancomycin, pristinamycin, chloramphenicol, linezolid, and Trimethoprim-sulfamethoxazole (SXT). PNSP (Penicillin non-susceptible pneumococci) was detected using an oxacillin disk (OXA 5 µg, <20 mm). The minimal inhibitory concentrations (MICs) of peni-

cillin G, amoxicillin, cefepime, and cefotaxime were determined using the BD Phoenix System following the manufacturer's protocol. The zone diameters of susceptibility testing were categorized as resistant, intermediate, or sensitive according to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2018).

Statistical analysis. Statistical analysis was done using the SPSS/PC 23.0 program (SPSS Inc., Chicago, IL, USA). Logistic regression analyses were performed to identify risk factors for pneumococcal carriage among AOM children. Statistical significance was determined with 95% confidence intervals (CIs). The differences were considered significant if the p-value was less than 0.05.

RESULTS

During the study period, 175 children were diagnosed with AOM. The patient group included 91 (52%) males and 84 (48%) females. The mean age of our study population was 21.41 months. Clinically, 167 of patients (95.4%) presented with fever, 83 (47.4%) with conjunctivitis, 43 (24.6%) with otorrhea, and 152 (86.9%) with otalgia. 25 (14.3%) had a history of prior AOM episodes. Bilateral AOM was recorded in 115 (65.7%) children. Of the total 175 AOM cases, 110 (62.9%) patients had been completely vaccinated, whereas 65 (37.1%) patients had received only two doses against *S. pneumoniae*. According to the questionnaire, 71 (40.6%) confirmed previous antibiotic treatment during the last three months.

Pneumococcal carriage in children with AOM. A total of 87 cultures were positive from the 175 samples collected, bringing the overall pneumococcal carriage to 49.7%. There was no significant difference in pneumococcal carriage between male and female groups (40/78, 51.2% and 47/97, 48.4% respectively, $p=0.710$). Young age was significantly associated with nasopharyngeal carriage of *S. pneumoniae* (6-11m: 35/58; 60.3%, 12-23m: 30/60; 50% and 24-36m: 22/55; 40%). Additionally, children having conjunctivitis exhibited lower pneumococcal colonization than normal children ($p<0.05$) (Table 1).

Pneumococcal serotypes and PCV serotypes identified in carriage isolates. Of the 63 pneumo-

coccal carriage isolates serotyped, 22 different serotypes were identified. The most frequent of these being 6C/6D (n=8, 12.7%), 10 (n=6, 9.5%), and 19B/19C (n=6, 9.5%), followed by 21 (n=5, 7.9%), 23B (n=5, 7.9%), 14 (n=4, 6.3%), 15B/15C (n=4, 6.3%), 7C/7B/40 (n=3, 4.8%), 11A/11D (n=3, 4.8%), 7F (n=2, 3.2%), 15A/15F (n=2, 3.2%), 17F (n=2, 3.2%), 19F (n=2, 3.2%), 35A (n=2, 3.2%), 39 (n=2, 3.2%), 1 (n=1, 1.6%), 3 (n=1, 1.6%), 4 (n=1, 1.6%), 9V (n=1, 1.6%), 13 (n=1, 1.6%), 20 (n=1, 1.6%), and 23A (n=1, 1.6%) (Fig. 1). Overall, vaccine serotypes (VT) comprised 19% (12/63) of which serotype 3 (n=1) was the only PCV13 serotypes, but not PCV10 serotypes, detected. On the other hand, non-vaccine serotypes (NVT) covered 81% (51/63) of the isolates. The most frequent NVT was 6C/6D (n=8) serotype.

Antibiotic resistance of *S. pneumoniae* isolates.

Antibiotic resistance was tested among the 40 available isolates. The prevalence rate of PNSP was 27.5%, where 27.2% of them were highly resistant to penicillin G (MICs ≥ 2 mg/ml) and 72.8% were intermediate (CMI>0.06-1 mg/l). The prevalence rate of non-susceptibility to amoxicillin, tetracycline, and erythromycin was 63.6%, 54.5%, and 10%. Among the all strains tested, antimicrobial susceptibility pattern showed a high rate of antibiotic susceptibility to levofloxacin, erythromycin, clindamycin, pristinamycin, and chloramphenicol, 39 (97.5%) isolates each. All isolates were susceptible to vancomycin (100%). The non-susceptibility rates to teicoplanin, tetracycline and trimethoprim-sulfamethoxazole were 7.5%, 15%, and 12.5% respectively (Table 2).

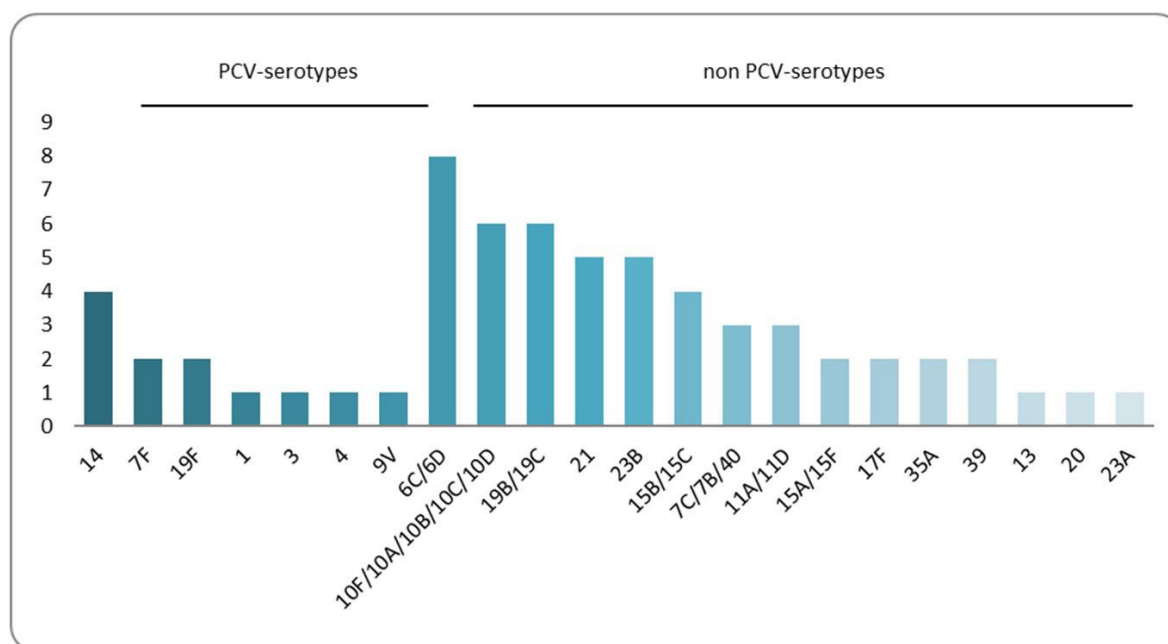
Serotype distribution of PNSP. Among VTs, serotype 19F was the only VT identified as PNSP, whereas serotype 14 was totally susceptible to penicillin G. In Contrast, PNSP was mostly associated with NVTs. Of these, 6C/6D, 11A/11D, 13, 17F, 21, and 23A were the most common serotypes in PNSP, accounting for 37.5%, 100%, 100%, 50%, 50%, and 100% (Fig. 2).

DISCUSSION

The nasopharynx is a natural host to many commensals, such as *S. pneumoniae*, that occasionally can become pathogenic (20-22). AOM infection is generally induced by otopathogens rise from the nasal cavity to the middle ear (23, 24). Many stud-

Table 1. Univariate and multivariate logistic regression analysis of *S. pneumoniae* carriage among AOM children

Characteristics	Colonized (n=87)	Non-colonized (n=88)	OR	95% CI	P value
Age (months)					
≤12	35	23			
13-24	30	30	1.522	0.733-3.158	0.260
25-36	22	35	2.421	1.145-5.121	0.021
Gender					
Male	40	38	1.058	0.785-1.426	0.710
Female	47	50			
Siblings	64	54	0.744	0.301-1.084	0,085
Preschool attendance	44	7	0.402	0.308-0.524	<0.001
Signs and symptoms					
Fever	83	80	1.528	0.677-3.448	0.240
Conjunctivitis	20	53	1.417	0.280-0.622	<0.001
Otorrhea	22	20	1.072	0.765-1.501	0.692
Otalgia	77	74	1.224	0.743-2.015	0.396
Bilateral AOM	65	52	0.694	0.501-0.961	0.028
Prior antibiotic use (<3 months)	46	53	0.861	0.640-1.158	0.326

**Fig. 1.** Serotype distribution of nasopharyngeal *S. pneumoniae* isolated from children with AOM in Marrakesh, Morocco

ies have reported the concordance between the nasopharyngeal carriage of *S. pneumoniae* and AOM (25-29). In this study of pneumococcal carriage in children suffering from AOM, the colonization rate of *S. pneumoniae* was 49.7%. Colonization rate of *S. pneumoniae* vary between studies from 40.5% to 68.3% (30-32). The colonization rate in the current study is in the middle of the reported range, as in this

study we recruited AOM children that are supposed to have an important colonization rate (33, 34). Ekinçi et al. (2021) demonstrated an overall pneumococcal colonization rate of 79.2% among AOM children after the introduction of PCV10 (26). Furthermore, Cohen et al. showed that the pneumococcal colonization prevalence has significantly reduced from 71.2% to 56.2% from 2001 to 2014 in France (35). Before

Table 2. Antimicrobial susceptibility rate of nasopharyngeal isolates among AOM children in Marrakesh, Morocco

Antimicrobial agents	N=40		
	Susceptible	Intermediate resistant	Resistant
Penicillin G	29 (72.5%)	8 (20%)	3 (7.5%)
Amoxicillin	33 (82.5%)	2 (5%)	5 (12.5%)
Cefepim	36 (90%)	1 (2.5%)	3 (7.5%)
Cefotaxime	33 (82.5%)	4 (10%)	3 (7.5%)
Tetracycline	34 (85%)	6 (15%)	-
Levofloxacin	39 (97.5%)	1 (2.5%)	-
Gentamycin-Syn	37 (92.5%)	3 (7.5%)	-
Teicoplanin	37 (92.5%)	3 (7.5%)	-
Vancomycin	40 (100%)	-	-
Erythromycin	39 (97.5%)	1 (2.5%)	-
Clindamycin	39 (97.5%)	1 (2.5%)	-
Pristinamycin	39 (97.5%)	1 (2.5%)	-
Chloramphenicol	39 (97.5%)	1 (2.5%)	-
Trimethoprim-Sulfamethoxazole	35 (87.5%)	5 (12.5%)	-

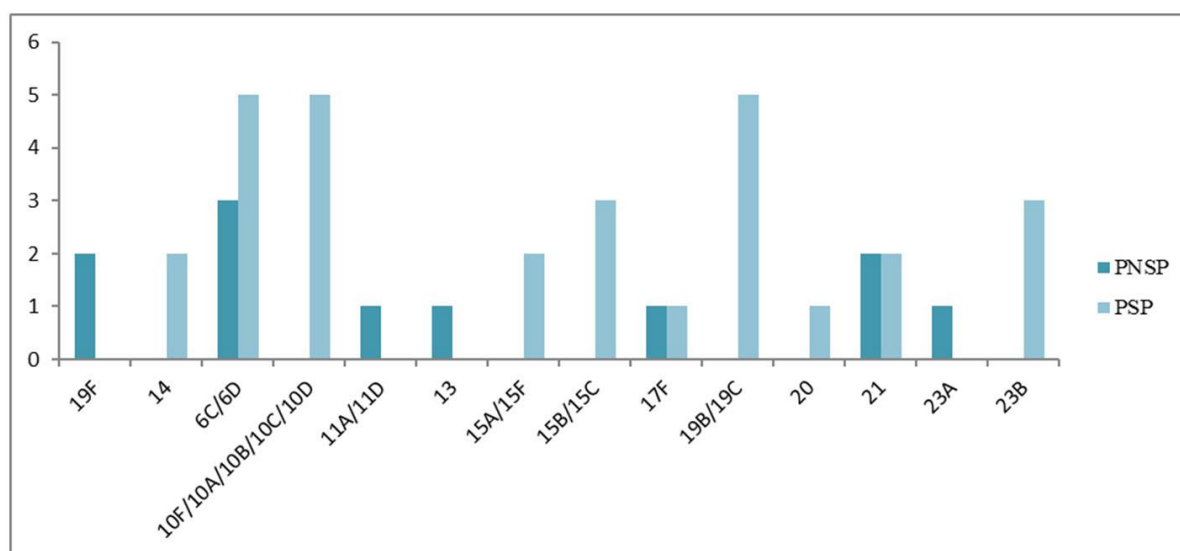


Fig. 2. Penicillin susceptibility of nasopharyngeal *S. pneumoniae* by serotype among children with AOM.

the introduction of PCV in Vietnam, *S. pneumoniae* colonization was about 35%, in 2016 (25). The Colonization rates of *S. pneumoniae* vary between studies according to many factors including population age, vaccination status, study period, inclusion criteria, and identification method (35-37).

After PCV13 was introduced to the Moroccan NIP in 2010, and was switched to PCV10 in July 2012, there was a significant drop in invasive pneumococcal disease where NVTs were mostly the major cause (31, 38). In our study, 19% of *S. pneumoniae* strains were VT, and 81% were NVT. These results provide

additional evidence for how vaccine can reduce VTs. Within this study, the most frequent serotypes were 6C/6D, 10, 19B/19C, 21, 23B, 15B/15C, 7C/7B/40, 11A/11D, 15A/15F, 17F, 35A, 39, 13, 20, and 23A. Of the evaluated non-PCV serotypes, 6C/6D was the most frequent, 10 and 19B/19C were the second, and 21 and 23B were the third most common serotypes. In Setchanova et al.'s study that involved 198 children with severe AOM, they showed that PCV10 coverage rate accounted for 40% (39). In Chi et al.'s study, the serotype coverage prevalence for PCV10 was 9.1% among patients aged 0 to 18 years (40).

Nevertheless, it is noticeable that VTs may be detected among AOM cases. In our study, we noted that all VTs were included in PCV10, except for serotype 3 which is included in PCV13. This is in line with Allemann et al.'s study that showed the persistence of serotype 3 even in the PCV 13 era (16). Similar to our data, a relatively important drop of VTs isolated from the middle ear specimens, and a significant increase in the prevalence of NVT was demonstrated after the generalization of PCV in Iceland (13).

Within this study, the frequency of PNSP was 27.5% among AOM children. Increasing rate of PNSP may be the main cause of failure of antibiotic treatment in AOM children (28). A high level of PNSP strains was reported by other authors in asymptomatic carriage and pneumococcal disease (29, 39, 41-43). Furthermore, 18.5% of *S. pneumoniae* strains were resistant to amoxicillin. This finding may be because penicillin G and amoxicillin are the first-line treatment. In addition, 90% and 82.5% of *S. pneumoniae* isolates are still susceptible to cefepime and cefotaxime. Our findings have confirmed recent reports confirming that PNSP was mostly associated with serotypes not targeted by the vaccine (44-46). Serotypes 6C, 19F, and 11A have been the subject of concern in many studies due to penicillin non-susceptibility (36, 41, 47). Further studies are recommended to comment on the clinical impact of antimicrobial resistance of these serotypes in Morocco.

The overall non-susceptibility prevalence of *S. pneumoniae* to erythromycin, clindamycin, pristinamycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole was 2.5%, 2.5%, 2.5%, 15%, 2.5%, and 12.5%, respectively. The non-susceptibility to erythromycin was lower than studies accomplished in Argentina (26.6%) (48), in Oman (28.1%) (49), in Iran (71.4%) (50), and in Taiwan (80%) (51). Two and a half percent (2.5%) isolates were resistant to clindamycin. Higher clindamycin resistance prevalences were observed in Oman (16.7%) (49), in Taiwan (77%) (51), and in Shanghai (97%) (52). Furthermore, 2.5% of our pneumococci strains were non-susceptible to pristinamycin, which is higher than a survey conducted in Morocco (0%) before introduction of pneumococcal vaccination (30). In addition, non-susceptibility to tetracycline was 15% in our study. Important non susceptibility prevalences were reported in Indonesia (44%) (53), in Iran (66.9%) (50), and in Taiwan (80%) (51). Furthermore, 2.5% of our pneumococci strains were non-suscep-

tible to chloramphenicol, which is comparable to 0.1% in Argentina (48), but lower than in Indonesia (9.7%) (54), and in Tanzania (18.4%) (55). Twelve and a half percent (12.5%) of our *S. pneumoniae* isolates were resistant to trimethoprim-sulfamethoxazol. High rates of non-susceptibility to trimethoprim-sulfamethoxazol was found in Indonesia (29.7%) (54), in Japan (37.9%) (56), in Iran (57.1%) (50), and in Shanghai (75.3%) (52). The rate of antimicrobial non-susceptibility differs greatly across countries depending on many factors such as age, nature of specimens, vaccination era, and antimicrobial consumption.

A limitation of this study was that we have only collected nasopharyngeal swabs from children suffering from AOM. We have not collected middle ear fluid specimens because of limited facilities available for the collection of middle ear fluid. Another limitation includes the fact that the enrollment in a private pediatric setting in Marrakesh, Morocco where the children enrolled might not represent the entire Moroccan pediatric population. In addition, similar research should be held, in different regions of Morocco, to evaluate the impact of pneumococcal vaccination against AOM.

CONCLUSION

In conclusion, the present study provides new baseline data on the nasopharyngeal carriage of *S. pneumoniae* among children suffering from AOM in Marrakesh, Morocco. It suggests that new NVTs are emerging, including 6C/6D and 10. Furthermore, it provides a relevant result, such as the spread of PNSP among AOM children. Pneumococcal carriage is an important determinant of the spread of new serotypes and antibiotic resistance, despite the efficacy of PCV10 in reducing the emergence of VT.

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