



## Serotypes and antibiotic susceptibility profile of encapsulated *Streptococcus pneumoniae* strains isolated from under five years old children

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### ABSTRACT

*Streptococcus pneumoniae* is one of the leading causes of childhood morbidity and mortality globally. The study determined the antibiotic susceptibility profile and molecular serotyping of encapsulated *S. pneumoniae* isolated from the blood of 100 under 5 years old children presented at the children's clinic of the Mother and Child Hospital Akure, Nigeria. The *S. pneumoniae* isolates were identified using standard microbiological techniques. Antibiotic susceptibility test included the Kirby-Bauer disc diffusion method. The detection of the strain serotypes by polymerase chain reaction (PCR) technique was conducted and the data derived from the study were statistically analysed. A total of 16 (16%) encapsulated *S. pneumoniae* strains were identified. All the 16 isolates were resistant to penicillin, cloxacillin, tetracycline and erythromycin. The isolates were all multi-resistant to the antibiotics tested. Serotypes 6B, 14 and 19F were detected. The study presents the baseline report of serotype 14 detection at the study centre. The continuous monitoring of serotype distribution and antibiotic susceptibility in this population will assist immensely in future preventive and treatment strategies.

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**Keywords:** *S. pneumoniae*, encapsulated, serotypes, blood, antibiotic resistance

### INTRODUCTION

*Streptococcus pneumoniae* is a bacterial pathogen that is an important cause of infections among children and the elderly (O'Brien et al., 2009; Hackel et al., 2013). The diseases caused by *S. pneumoniae* range from mild respiratory tract non-invasive mucosal infections such as otitis media and sinusitis to more severe invasive diseases such

as pneumonia and meningitis (Henriques-Normark and Tuomanen, 2013). Among the virulence factors of *S. pneumoniae* is the polysaccharide capsule that prevents phagocytosis by interfering with binding of complement V3b to the cell's surface by the host's immune cells (Barocchi et al., 2006; Kadioglu et al., 2011). *S. pneumoniae* and *Haemophilus influenzae* have been reported to

be the leading causes of acute respiratory infections in children in developing countries (O'Brien et al., 2009).

The organism has been observed to be the cause of childhood mortality from vaccine preventable illness especially in under five years old children worldwide (O'Brien et al., 2009; Apansia et al., 2015). In Nigeria it has been observed that *S. pneumoniae* is the cause of 13% of overall deaths in north Central Nigeria and 80% of deaths from meningitis in Ibadan both occurring in children below 5 years old (Falade et al., 2009; Obaro et al., 2011; Iwalokun et al., 2012). The risk factors for invasive pneumococcal disease (IPD) in Nigeria identified includes air pollution overcrowding nasopharyngeal carriage and high level transmission of the pathogen as well as the presence of co-morbidities such as HIV/AIDS and sickle cell anaemia (Adetifa et al., 2012).

Of all the ninety-one pneumococcal serotypes that have been described, only 46 have been categorised into serogroups based on their immunological cross-reactivity and not all have been observed to cause diseases (Hausdroff et al., 2000; Shouval et al., 2006; Park et al., 2007). Reports have shown that not all virulent serotypes and majority of antibiotic resistant strains have been covered in the conjugate vaccines in use (Nuorti et al., 2010; Gant et al., 2012; Pichon et al., 2013).

The emergence of multiple antibiotic resistant strains has been observed to complicate the management of infections caused by *S. pneumoniae* (Beekman et al., 2005). It has been reported that there is a correlation between serotypes and antimicrobial resistance (Hecini-Hannachi et al., 2014). However, Link-Gelles et al. (2013) suggested selective pressure from antibiotics use determines resistance patterns rather than

differences in serotype distribution. The aim of the present study was to determine the effect of serotype distribution and antibiotic susceptibility pattern of *S. pneumoniae* strains isolated from paediatric population in the study centre.

## MATERIALS AND METHODS

### Ethical clearance and samples collection

A total of 100 under five years old children presented at the children's clinic of the Mother and Child Hospital in Akure, Nigeria were recruited for the study. All were consecutively recruited after ethical clearance and informed consent were obtained. Two ml venous blood were aseptically collected from each subject and transferred immediately into culture bottles containing sterile Robertson's cooked meat broth (Biomark, India) and incubated at 37 °C for 18 h.

### Isolates identification

A turbid appearance in the culture bottles indicated the growth of microorganisms. A loopful of the culture was thereafter, streaked on a sterile blood agar plate (blood base agar enriched with 5% sheep blood). The plates were inverted and placed in a CO<sub>2</sub> jar and incubated at 37 °C for 24 h. The isolates were identified as *Streptococcus pneumoniae* using methods such as catalase, capsule, optochin susceptibility test as described (Ruoff et al., 2003).

### Antibiotic susceptibility testing

The Kirby-Bauer disc diffusion was used to test for the antibiotic susceptibility of the isolates and the isolates were standardised to 0.5 McFarland standard. The impregnated susceptibility discs of known concentrations included penicillin G (10 units), amoxicillin/clavulanic acid (2:1) (Oxoid, UK)

while cefuroxime (30 µg), gentamicin (10 µg), ceftriaxone (30 µg), ofloxacin (5 µg), cotrimoxazole (25 µg), erythromycin (5 µg), tetracycline (30 µg), chloramphenicol (5 µg) ceftazidime (30 µg), amoxicillin (5 µg) and cloxacillin (5 µg) (Abtek Biologicals, UK). The isolates were inoculated onto Mueller Hinton agar containing 5% sheep blood, antibiotics disc were aseptically placed and incubated at 37 °C in a candle jar for 18 h. The plates were thereafter, read and susceptibility was interpreted according to the approved guidelines of the CLSI for *Streptococcus pneumoniae* (CLSI, 2013) and AB Biodisk interpretative chart. Resistance of the isolates to three or more of the following antibiotic classes: penicillin, erythromycin, chloramphenicol, tetracycline or trimethoprim-sulfamethoxazole (cotrimoxazole) was referred to as multi-drug resistant.

#### **Preparation of DNA template**

The DNA of the isolates was extracted as described (CDC, 2011). Bacterial colonies were suspended in 0.85% normal saline and briefly vortexed and incubated at 70 °C for 15 minutes. The suspension was subsequently centrifuged at 12000 x g for 2 minutes and the supernatant was decanted. The cells were resuspended in 50 µl TE buffer containing 10 µl mutanolysin and 8 µl hyaluronidase. The samples were incubated at 37 °C for 18 h and

the suspension was boiled for 10 minutes and finally centrifuged at 12000 x g for 4 minutes. The supernatant obtained was used as the template.

#### **Detection of the serotypes**

The serotypes 6B, 14, 7F, 9V, 18 and 19F detection with published primer sequences and polymerase chain reaction (PCR) method were done as described (CDC, 2011) and the serotype primer sequence are as shown in Table 1. The sequential PCR reactions for a 25 µl included 0.5 µl forward and reverse primers, PCR grade water (17 µl) and taq polymerase (5 µl) that made an aliquot of 23 µl. The 23 µl master mix was aliquoted into PCR eppendorf tube and a 2 µl of DNA lysate was added to each appropriate tube making a total of 25 µl reaction. The PCR tubes were loaded in the GeneAmp PCR system 9700 thermocycler (Applied Biosystems) and the amplification and cycling steps are as described (CDC, 2011). The PCR product was electrophoresed on a 1% Tris Borate EDTA (TBE) agarose gel at 75V for 60 secs. The bands were subsequently visualised under UV transilluminator and photographed with a digital camera.

Simple percentage/mean were used to present the frequency of the *Streptococcus pneumoniae* isolates and the antibiotics susceptibility patterns.

**Table 1:** Primers used for the detection of the target genes.

Primers	Primer sequence (5 <sup>1</sup> -3 <sup>1</sup> )	Gene	PC (μM)	Bp	NOC	Denaturation	Annealing	Extension	Reference/Source
6A/6B/6C/6D	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	<i>wciP</i>	0.3	250	35	94 °C for 30s	57 °C for 45s	72 °C for 40s	CDC, 2011
6A/6B/6C/6D	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA								
7F/7A	TCC AAA CTA TTA CAG TGG GAA TTA CGG	<i>Wzy</i>	0.4	559	35	94 °C for 40s	61 °C for 60s	72 °C for 60s	CDC, 2011
7F/7A	ATA GGA ATT GAG ATT GCC AAA GCG AC								
9V/9A	GGG TTC AAA G TC AGA CAG TG A ATC TTA A	<i>wzy</i>	0.5	819	35	94 °C for 40s	60 °C for 45s	72 °C for 45s	CDC, 2011
9V/9A	CCA TGA ATG A AA TCA ACA TT G TCA GTA GC								
14	GAA ATG TTA CTT GGC GCA GGT GTC AGA ATT	<i>wzy</i>	0.3	189	40	94 °C for 40s	58 °C for 45s	72 °C for 45s	CDC, 2011
14	GCC AAT ACT TCT TAG TCT CTC AGA TGA AT								
18/(18A/18B/18C/18F)	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC	<i>wzy</i>	0.3	573	40	94 °C for 60s	58 °C for 45s	72 °C for 45s	CDC, 2011
18/(18A/18B/18C/18F)	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC								
19F	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	<i>wzy</i>	0.5	304	35	94 °C for 40s	58 °C for 45s	72 °C for 45s	CDC, 2011
19F	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG								

**RESULTS**

A total of 16 (16%) encapsulated *S. pneumoniae* isolates were identified. All the isolates were resistant to penicillin, amoxicillin, cloxacillin, erythromycin and tetracycline, while 13 (81.3%) of the isolates were resistant to cotrimoxazole. Nine (56.3%) of the isolates were resistant to Amoxicillin/Clavulanate 2:1, ceftazidime and chloramphenicol while 7 (43.3%) of the isolates were resistant to cefuroxime and 2 (12.5%) to ceftriaxone and gentamycin. However, 15 (93.8%) of the isolates were susceptible to ofloxacin as shown (Table 2).

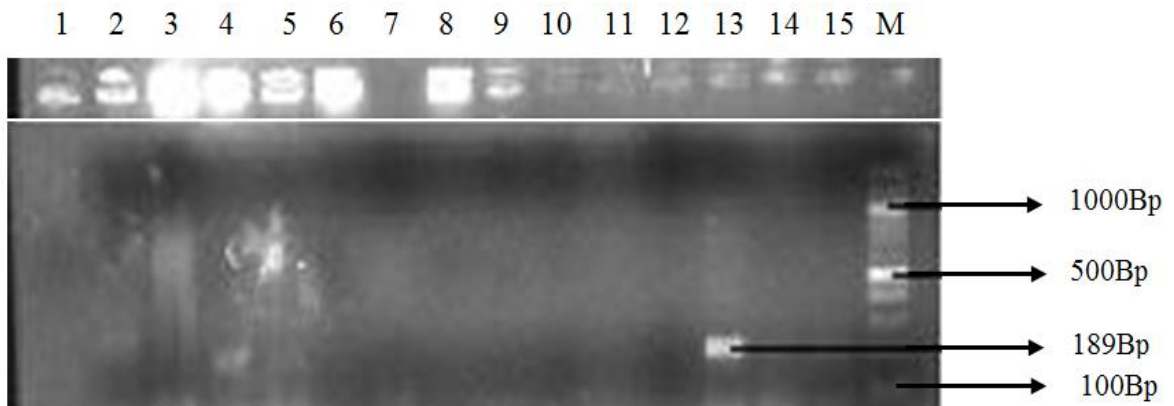
Of the 16 isolates serotyped, eleven of the isolates were typable while 5 were non-

typable. The serotypes detected were serotypes 19F which accounted for 6 of all the serotypes recovered followed by 4 isolates for serotype 6B. Serotype 14 was detected in only one of the isolates at 189bp (Figure 1).

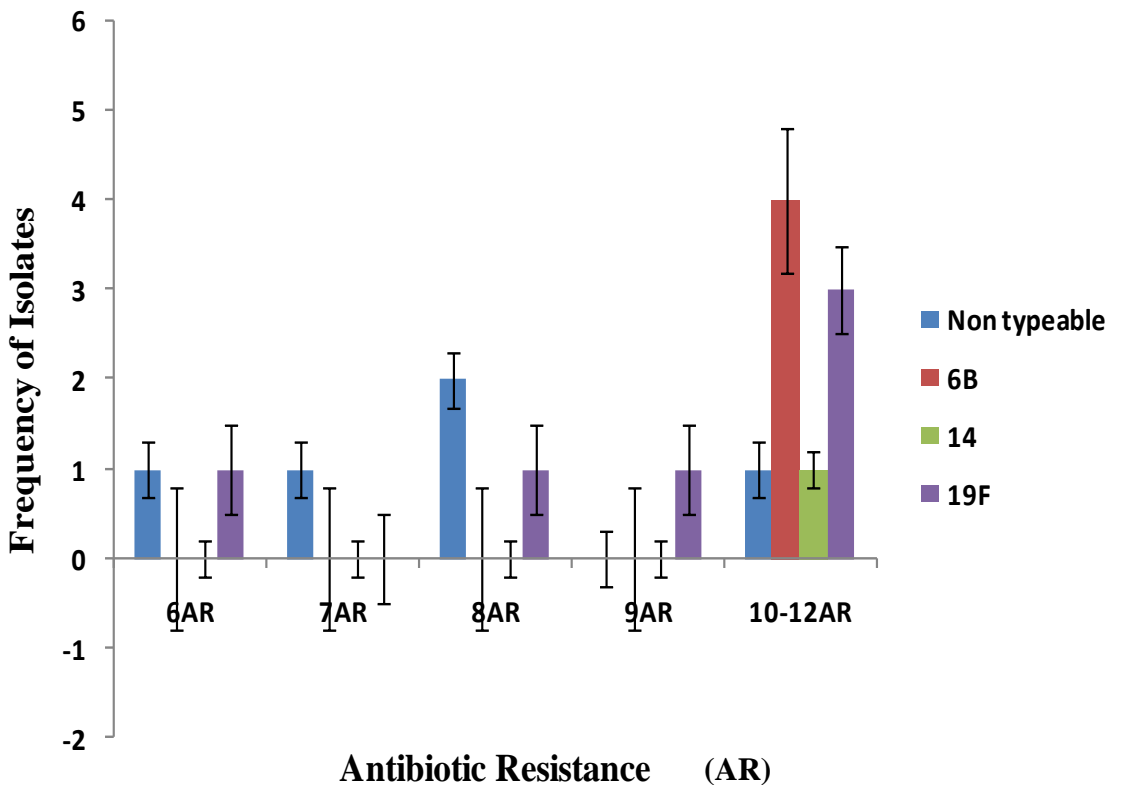
The multiple antibiotic resistance of the 16 isolates showed that all the typable and non- typable strains were multiple antibiotic resistant. In all, the isolates were resistant to three or more classes of antibiotics. The three serotypes were resistant to 6-12 antibiotics; one was susceptible to 4 antibiotics but six were susceptible to 5-7 antibiotics while nine isolates were resistant to 10-12 antibiotics. It was observed that serotypes 14 and 6B were resistant to 10-12 antibiotics (Figure 2).

**Table 2:** Profile of antibiotic susceptibility of the *S. pneumoniae* isolates.

Antibiotics (µg)	Number of isolates	Susceptible	Intermediate Number (%)	Resistant
Amoxicillin (25 µg)		0	0	100
Amoxicillin/Clavulanate 2:1		4 (25)	3 (18.8)	9 (56.3)
Ceftazidime (30 µg)		4 (25)	3 (18.8)	9 (56.3)
Ceftriaxone (30 µg)		7 (43.8)	7 (43.8)	2 (12.5)
Cefuroxime (30 µg)		6 (37.5)	3 (18.8)	7 (43.8)
Chloramphenicol (30 µg)	16	5 (31.3)	2 (12.5)	9 (56.3)
Cloxacillin (5 µg)		0	0	100
Cotrimoxazole (30 µg)		1 (6.3)	2 (12.5)	13 (81.3)
Erythromycin (5µg)		0	0	100
Gentamycin (10 µg)		13 (81.3)	1 (6.3)	2 (12.5)
Ofloxacin (5 µg)		15 (93.8)	1 (6.3)	0
Penicillin G (10 units)		0	0	100
Tetracycline (30 µg)		0	0	100



**Figure 1:** PCR detection of serotype 14 in *S. pneumoniae* isolates. Lane 13 is positive for serotype 14 as indicated by 189bp. Lane M is the molecular weight size marker.



**Figure 2:** Serotypes and antibiotic resistance pattern of the isolates.

**DISCUSSION**

The study considered the antibiotic resistance and the serotypes of *S. pneumoniae* isolated from hospitalised children and determined the relationship between serotype distribution and antibiotic susceptibility. In this present study, the encapsulated *S. pneumoniae* identified were all resistant to penicillin and were multi-drug resistant. The findings reported by previous authors (Falade et al., 2009; Iwalokun et al., 2012) with the result obtained from this work have demonstrated changing trend in antibiotic susceptibility among the circulating serotypes of *S. pneumoniae*, in Nigeria. A gradual increase has been observed in resistance level among antibiotics that were initially effective against the organism.

It has been reported that patterns of change in serotype distribution varies among countries and regions (Hecini-Hannachi et al.,

2014; Huang et al., 2015). Serotype 14 has been described as one of the most common serotype causing invasive pneumococcal disease in children less than five years old children (Imöhl et al., 2010, Hecini-Hannachi et al., 2014). However, in studies on *S. pneumoniae* serotypes distribution in Nigeria there has been no known report of serotype 14 (Falade et al., 2009; Adetifa et al., 2012; Iwalokun et al., 2012). In this present study the only serotype 14 *S. pneumoniae* detected was resistant to erythromycin, tetracycline and the  $\beta$ -lactam antibiotics. Studies have shown that serotype 14 is frequently resistant to erythromycin (Clarke, 2006; Ihekweazu et al., 2007).

Apart from the serotype 14 *S. pneumoniae* strains identified, two other distinct serotypes 6B and 19F were also detected in this study. Serotype 19F is the most predominant and correlates with recent

study in China on serotype distribution in children (Huang et al., 2015). All the serotypes including the non-typable strains were multi-antibiotic resistant and this agrees with the report of Apansia et al. (2015). The isolates were highly resistant to the  $\beta$ -lactams antibiotics, tetracycline, cotrimoxazole and erythromycin, but low resistance was observed to gentamycin and ofloxacin. In a hospital-based study conducted in Ilorin, Nigeria in 2002, Akanbi et al. (2004) reported the recovery of *S. pneumoniae* isolates that were resistant to all tested antibiotics, including penicillin, erythromycin and ceftriaxone. The high multi-drug resistance observed in the isolates emphasises the need for surveillance. In addition, early and strict compliance to vaccine regimen will ensure protection against pneumococcal infection. Also appropriate antibiotics use has been suggested will address antibiotic resistance in *S. pneumoniae* (Gertz et al., 2010).

Pneumococcal serotypes vary in their likelihood of carriage, invasive potential, and disease severity which may be related to resistance (Ramdani-Bougoussa and Rahal, 2003). It has been posited that factors that alter serotype distribution may influence the prevalence of antibiotic resistance and co-colonization may also increase the likelihood of exchange of genes causing mutation that may lead to acquisition of resistance (Song et al., 2012). However, further study with large number of isolates and varied serotype is suggested since isolates size and serotypes analysed in this study is small.

### Conclusion

The study provides important baseline data for continued surveillance of antibiotic resistance and serotype changes in this population that will assist immensely in future preventive and treatment strategies.

### COMPETING INTERESTS

The authors declare that they have no competing interest.

### AUTHORS' CONTRIBUTIONS

This work was conducted in collaborations between all the authors.

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