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Molecular Identification and Multidrug Resistance Pattern of Clinical Streptococcus pneumoniae Isolate

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ABSTRACT:

A frightening increase in infections due to resistant pneumococci has been documented in nearly all countries and a growing number of clinical failures following the use of antibiotics have been reported. This study seeks to carry out molecular identification and evaluate multidrug resistance pattern of *Streptococcus pneumoniae* isolated from a hospital in Calabar, Nigeria. One hundred and fifteen (115) clinical specimens (sputum, blood, ear swab, urine and catheter) were sampled from volunteer patients in the University of Calabar Teaching Hospital, Calabar. Thesamples were analyzed for the presence of *S. pneumoniae* using standard bacteriological methods. Molecularly identified isolate was subjected to antimicrobial susceptibility testing using disc diffusion technique.Result indicates that a total of ninety-eight (98) *Streptococcus* species were isolated in this study. Thirty-nine (39.80%) were recovered from sputum, 12 (12.24%) from blood, 16 (16.33%) from ear swab, 10 (10.20) from urine and 21 (21.43) were obtained from catheter. Susceptibility test result revealed resistance of *Streptococcus pneumoniae* to ampicillin, amoxillicin, cloxacillin, gentamycin, amikacin, and erythromycin.There was no statistical significant difference amongst the zones of inhibition at ($P \leq 0.05$).This study has demonstrated the presence and multiple drug-resistance of *S. pneumoniae*in Calabar, Southern Nigeria. We, therefore, suggest that proper antibiotics use should be adopted to avert treatment failure consequences.

KeyWords: **Streptococcus pneumoniae, resistance, clinical, multidrug, patients**.

I INTRODUCTION

The genus *Streptococcus* belongs to the non-spore forming aerobic to facultative anaerobic bacteria. They are Gram-positive, occurring in pairs or in chains (Iroha *et al.,* 2012). *Streptococcus pneumoniae* can induce diverse spectrum of diseases associated with considerable morbidity and mortalityespecially in children, the elderly and the immunocompromised. Pneumococci are the leading cause of community-acquired pneumonia and a very frequent cause of pneumonia, otitis media, sinusitis, bacteraemia and meningitis (Akanbi *et al*., 2018).

Strains of *S. pneumoniae* were usually considered to be sensitive to β-lactam drugs before the 1960s and routine antibiotic susceptibility testing were not performed in hospitals (Iliyasu *et al*., 2015). The first clinically significant isolate not susceptible to penicillin was reported in 1967 from

Australia (Kandakai-Olukemi and Dido, 2009). Since then penicillin-resistant *S. pneumoniae* has been reported worldwide with increasing frequency and multiple antibiotic resistant strains have also been reported (Agwu *et al*., 2014; Livni *et al*., 2013; Frindland and McCraken, 2017).

Increasing antimicrobial resistance amongst previously susceptible bacterial species to frequently used antibacterial compounds has constituted a global health challenge (Thualfakar*et al*., 2020). Pneumococcal resistance to antibiotics evolves due to structural modification or alteration in the penicillin binding proteins (PBPs). It is pertinent to note that resistance to β-lactam drugs by most bacteria is as a function of the activity of β-lactamase enzyme that is capable of hydrolysing these antibiotics; but resistance in *S. pneumoniae* to penicillin and other β-lactams is due to the expression of low affinity PBPs and not β-lactamase production (Zhang *et al*., 2014). In these resistant isolates, there has been a reduction in the affinity of at least three of the five high molecular weight PBPs found in this bacterium (Akanbi *et al*., 2018).

The antimicrobial resistance pattern of *S. pneumoniae* has been reported in many countries. For instance, moderate range of resistance has been publicized in the United States and Canada (Wierzbowski *etal*., 2017; 2019). One major risk factor that is associated with the development of drug resistance by pneumococci is prior antibiotic use (Kiran *etal*., 2016). Here in Nigeria, information on the resistance pattern of *S. pneumoniae* is scarce, hencethe current study is undertaken to identify and evaluate the multidrug resistance pattern of *Streptococcus pneumoniae* isolated from clinical specimens sampled at University of Calabar Teaching Hospital, Calabar, Nigeria.

II MATERIALS AND METHODS

A. Study site and study population

This study was conducted from May 2021 to September 2021 after clinical samples were collected from University of Calabar Teaching Hospital (UCTH), Calabar, Nigeria. The study population consisted of 115 patients. A total of 115 samples were collected comprising of 23 each of specimens (sputum, blood, ear swab, urine and catheter). All participants were clinically diseased with respiratory tract infections (cough), bacteremia, otitis media, and urinary tract infections (UTIs), and using antibiotics at the time of sample collection. Informed consent was obtained from each patient, and from parents or guardians on behalf of children participants involved in the study before enrollment. Ethical clearance was obtained from the Health Research Ethics Committee ofUCTH, Calabar. This was used to collect clinical specimens from patients in the hospital.

B.Specimen collection

Clinical specimens were collected using sterile cotton swabs, small screw capped bottles, a firmly stopper tube, syringe and a sealed capillary tube (Koneman *etal*., 2005). The specimens collected include urine, ear swabs, sputum, blood and catheter as sample from hospital environment. Each swab was carefully taken from the site of infection and placed in tubes containing readymade media (transport media - nutrient broth with 5% sheep blood added) to maintain the swab wet during transportation to laboratory.

C. Culture of specimen and identification of bacteria

Specimen was cultured immediately on blood agar (augmented with 5% sheep blood) and incubated at 37°C for 24 hrs. After incubation, colonies which appeared small, grey, moist with characteristic zone of alpha-haemolysis (green) was further identified by biochemical tests (such as Gram stain, motility, oxidase, catalase, urease, methyl red, Voges Proskauer, optochin sensitivity test, bile solubility test and molecular analysis using 16S rRNA gene sequencing by Sanger method(Cheesbrough, 2000; Nishiguchi *et al*., 2000; Trindale *et al*., 2007).

D. Molecular identification of bacterial isolates

(i) Genomic DNA extraction procedure

Reagents used include:

1. 5M NaCl- 29.22 gm for 100 ml (Autoclaved)
2. SDS 20%- 10 gm for 50 ml (pH-7.2)
3. CTAB (10%) /NaCl (0.7M) Solution- 4.09 gm for 100 ml NaCl and 10 gm of CTAB (Autoclave)
4. Chloroform: Isoamyl alchohol – 24:1
5. TE (Autoclave)

Procedure:

Liquid cultures (1-3 mL) were centrifuged at 4600x *g* for 5 min. The resulting pellets were re-suspended in 520 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were added. The mixture was incubated for 1 hr at 37 ºC. After incubation, 5 M NaCl (100 µl) and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and mixed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifuged at 7200 x *g* for 20 min. The aqueous phase was transferred to a new tube, isopropanol (1:0.6) was added and DNA was precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 7200 x *g* for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours.Pellets were re-suspended in 50 µl of TE buffer and kept at 4°C (Nishiguchi *et al*., 2000; Trindale *et al*., 2007).

(A) Protocol for bacterial polymerase chain reaction (PCR)

- **Primers**

1. Forward primer: 27f (5'-AGAGTTTGATCMTGGCTCAG-3')
2. Reverse primer: 1525r (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991).

- **Cocktail reaction**

Ingredient	Volume (µl)
1. Sterile distilled H_2O	6.44
2. 5 U /µl *Taq* Polymerase	0.06
3. 25 mM $MgCl_2$	0.75 (1.5 mM)
4. 10 mM dNTPs	0.25 (250 µM)
5. 10 pM forward primer	0.25 (1 µM)

6. 10 pM reverse primer 0.25 (1 µM)
7. Template 2
8. Total volume 12.5

- **PCR conditions**
  1. 94°C for 2 mins
  2. 30 cycles of 94°C for 30 secs, 50°C for 60 secs and 72°C for 90 secs
  3. 72°C for 5 mins
  4. Store at 4°C
- **Post PCR analyses**

To make agarose gel, 1.5 g of agarose powder was added into 100 ml of 1X TAE Buffer. Heated in a microwave for 5 minutes.Cooled briefly and 5 µl of GR Green® solution was added.Mixed briefly and poured into a gel tank with well combs.The mixture was left to solidify and PCR products was loaded into each well.Electrophoresis (Gel-Field) was performed at 100V for one hour.Gel was viewed under UV light and pictures were taken.Expected product size: 1,500 bp.Sequencing was done using purified amplicons. Fragments were sequenced using Nimagen, BrilliantDye^{TM}Terminator sequencing Kit V3.1 BRD3-100/1000 according to manufacturer's instructions.The nucleotide of the isolates were subjected to nucleotide BLAST analysis to reveal their percentage identity/similarities, accession number, highest query cover (%), E value, number of nucleotides and verification of amplification of polymerase chain reaction.

E. Construction of phylogenetic tree

Phylogenetic tree showing evolutionary relationship of *Streptococcuspneumoniae* isolated from University of Calabar Teaching Hospital, Calabar was constructed using the methods described by Jukes and Cantor (1969), Felsenstein, (1985), Saitou and Nei (1987) and Tamura *et al*. (2013).

F. Test for antibacterial resistance pattern using antibiotics

The identified bacterial isolate was evaluated for resistance to a panel of 15 of antibiotics using Kirby Bauer disk diffusion method (Akubuenyi *et al*., 2018; Otu, 2020). The concentrations of antimicrobial sensitivity testing discs that were used and interpretation of sizes and zones of inhibition were in agreement with performance standards for antimicrobial disc susceptibility tests (CLSI, 2020). Susceptibility of isolates to antibiotics was determined on Mueller-Hinton agar plates containing 5% sheep blood. Susceptibility tests were performed from a bacterial inoculum whose turbidity was equivalent to that of a McFarland standard of 0.5. The inoculum was produced from a pure culture of the isolate to be tested. The inocula were spread over the surface of the agar plates and the antibiotic (Antec Diagnostics, UK) discs were placed on the plates and incubated at 37°C for 24 hrs. The diameter of the zone of inhibition for each test antibiotic was measured and sensitivityor resistance estimated by comparing with zone-diameter interpretive standard. Antibiotics tested were ciprofloxacin (10mcg), chloramphenicol (30mcg), gentamicin (30mcg), streptomycin (30mcg), erythromycin (10mcg), ampicillin (10mcg), amikacin (10mcg), levofloxacin (30mcg), ceftazidime (20mcg), cloxacillin (10mcg), septrin (30mcg), oxacillin (10mcg), amoxillin (10mcg), augmentin (30mcg) and ciprofloxacin (20mcg). Multidrug resistance bacteria was taken as resistance to one drug in three or more groups of antibiotics (CLSI, 2017; CLSI, 2020).Five (5) classes of antibiotics used include:

1. **Penicillins:** Ampicillin, Cloxacillin, Amoxicillin, Oxacillin and Augmentin
2. **Aminoglycosides:** Gentamycin, Streptomycin, Amikacin, Chloramphenicol

3. **Guinolones:** Ciprofloxacin, Levofloxacin, Norfloxacin.
4. **Macrolides**:Erythromycin
5. **Sulphonamides:** Septrin
6. **Cephalosporin:** Ceftazidime

G. Statistical analysis

All the statistical analyses were performed by using the SPSS statistical package version 21.0 (SPSS, Chicago, Illinois, USA). Analysis of variance was performed by comparing the zones of inhibition recorded. P value less than or equal to 0.05 were considered statistically significant.

III RESULTS

A total of ninety-eight (98) *Streptococcus* species were isolated in this study from different specimens. Thirty-nine (39.80%) were recovered from sputum, 12 (12.24%) from blood, 16 (16.33%) from ear swab, 10 (10.20) from urine and 21 (21.43) were obtained from catheter. All isolates were associated with clinical infections. The result is presented in Table 1.Morphological studies revealed that all of the isolates gave small (0.5mm), circular, smooth, grey and lancet colonies on blood agar plates. All isolates were Gram positive, cocci, non-motile, catalase and oxidase negative, methyl red positive, Voges-Proskauer and urease negative. They grew in the presence of 5% CO_2, 40% bile salts, pH 8.4 and at 35°C to 37°C (Table 2). Based on the morphological, physiological and biochemical characteristics, all the isolates were tentatively identified as *Streptococcus* species. Among them, 1 isolate was selected for further molecular and antibiotic studies. The 16S rRNA gene sequence data of the isolate exhibited 99.93% homology with *Streptococcus pneumoniae* strains obtained from database. The sequence of the isolate has been deposited to the National Center for Biotechnology Information(NCBI) GenBank with accession number:MZ802726 (Table 3).

Plate 1 shows the Gel Image of polymerase chain reaction (PCR) of *Streptococcuspneumoniae* isolate.Phylogenetic tree showing evolutionary relationship of *Streptococcus pneumoniae*obtained from UCTH-Nigeria (in red) with others stains available in the GenBank database is presented in Figure 1.The optimal tree with the sum of branch length = 0.00562181 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1428 positions in the final dataset.

Table 1: Percentage (%) prevalence of *Streptococcus* speciesisolated from clinical specimens

S/N	Specimen	Occurrence of *Streptococcus* sp.	% occurrence of *Streptococcus* sp.
1.	Sputum	(23) 39	39.80
2.	Blood	(23) 12	12.24
3.	Ear swab	(23) 16	16.33
4.	Urine	(23) 10	10.20
5.	Catheter	(23) 21	21.43
Total		**115 98**	**100**

Table 2: Biochemical test and preliminary identification of *Streptococcus* species

Test type	Tests	Characteristics
Colonial characters	Size	0.5mm (blood agar)
	Type	Round
	Colour	Grey, mucoid (blood agar)
	Shape	Lanceolate
Morphological characters	Motility	-
	Shape	Cocci (diplococci)
	Spore	Non-spore forming
	Growth at 35-37^{o}C	+
Physiological characters	Growth in 40% bile salt	+
	Growth in 5% CO_2+	
	Growth in sheep blood	+
Biochemical characters	Gram staining	+
	Catalase	-
	Oxidase	-
	Urease	-
	VP	-
	MR	+
	Haemolysis	+ (alpha haemolysis)
	Optochin test	Sensitive

VP: Voges Proskauer, MR: Methyl Red, +: Positive, -: Negative: mm: Millimetre

To find out whether the identified *Streptococcus pneumoniae* isolate had resistance against commercial antibiotics, we screened them against 15 antibiotics using disk diffusion assay. Interestingly, the bacterium displayed resistance to multiple antibiotics viz., ampicillin (10mm), amoxillin (11mm), cloxacillin (13mm),gentamycin (14mm), erythromycin (9mm) and amikacin (12mm) (Table 4). However, these isolates exhibited varying levels of susceptibility to oxacillin (19mm), augmentin (25mm), chloramphenicol (26mm), ciprofloxacin (19mm), levofloxacin (24mm) and ceftazidime (19mm). In addition, the organism was intermediate to streptomycin (15mm) and septrin (14mm).There was no statistical significant difference amongst the zones of inhibition at ($P \leq 0.05$).

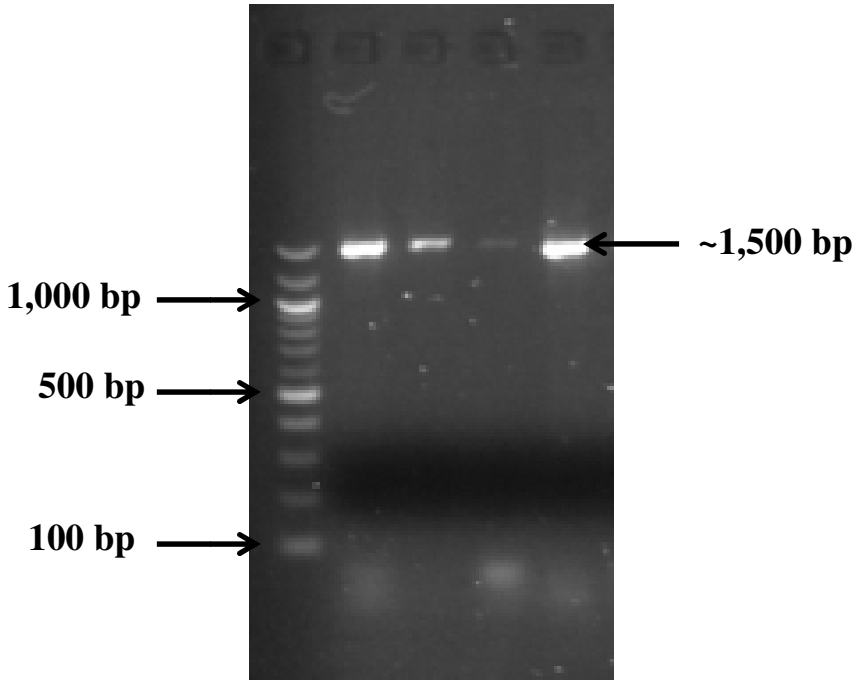

Plate 1: Gel Image of polymerase chain reaction (PCR) of *Streptococcus pneumoniae*

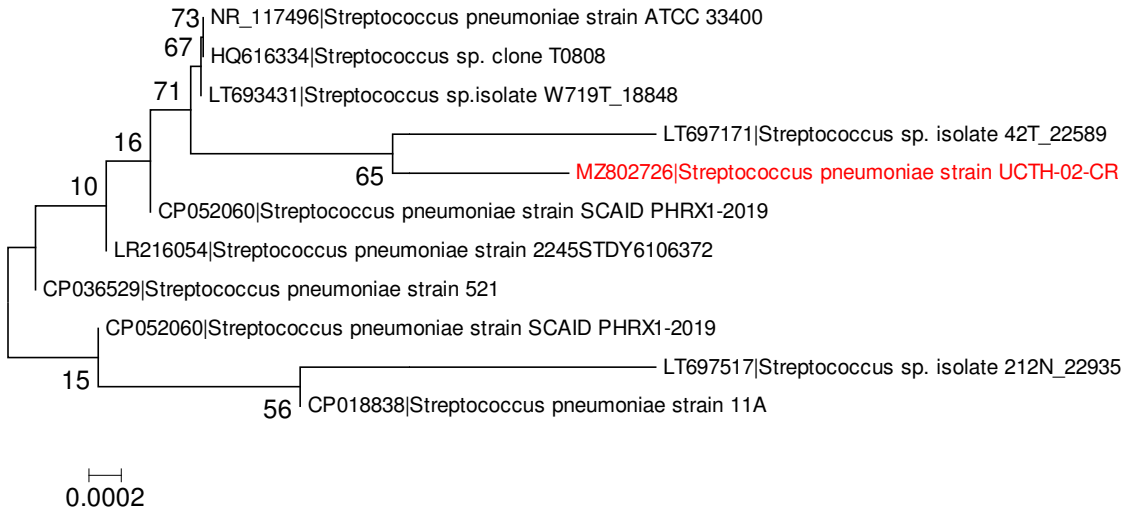

Figure 1: Phylogenetic tree showing evolutionary relationship of *Streptococcus pneumoniae*obtained from UCTH-Nigeria (in red) with others stains available in the GenBank database.

Table 3: Characteristics of partial 16S ribosomal RNA sequence of *Streptococcus pneumoniae* isolated at the University of Calabar Teaching Hospital, Calabar, Cross River State, Nigeria.

Sample/organism	Strain	Accession no.	No. of Nucleotides	Highest nBLAST score	Highest identity (%)	E value	Alignment coverage (%)	Highest query
Streptococcus pneumoniae	UCTH-02-CR	MZ802726	741	99.33		0.00	≥200	100

BLAST: basic local alignment search tool, n: nucleotides, %: percentage

Table 4: Antibacterial susceptibility of *Streptococcus pneumoniae*isolated from clinical specimen

Antibiotic agents	Zones of inhibition (millimetre)	
Ampicillin 10(R)	I: Intermediate	
Amoxicillin 11(R)	R: Resistance	
Cloxacillin 13(R)		S: Sensitive
Oxacillin 19(S)		
Augmentin 25(S)		
Gentamycin 14(R)		
Streptomycin 15(I)		
Amikacin 12(R)		
Chloramphenicol 26(S)		
Ciprofloxacin 19(S)		
Levofloxacin 24(S)		
Norfloxacin 11(R)		
Erythromycin 9(R)		
Septrin 14(I)		
Ceftazidime 19(S)		

Percentage resistance of *S. pnuemoniae* to tested drugs is 46.66%.

IVDISCUSSION

The way drugs are prescribed by charlatans in the health-care system, under dosing, over prescription and outright fake drug racketeering has become profound in Nigeria (Arikpo *et al*., 2011; Akpede and Abiodun, 2012). These factors lead to development of resistance by bacteria to drugs that otherwise would have aided the treatment of infections (Iliyasu *et al*., 2015). Our investigation revealed pneumococcal resistance to commonly used antibiotics in a tertiary referral centre (UCTH) in southern Nigeria (Table 4).

The present study showed *Streptococcus pneumoniae* resistance to penicillin derivatives (ampicillin, amoxicillin and cloxacillin).This finding is consistent with other studies, which have reported the resistance of *S. pneumoniae* to penicillins and its derivatives (Kandakai-Olukemi and Dido, 2009; Iroha *et al*., 2012; Iliyasu *et al*., 2015). Several factors have been reported to be related to carriage and transmission of resistant*S. pneumoniae*. However, the most important factor is probably recent antibiotic use. Previous antibiotic exposure has been documented as a risk factor for antibiotics resistance in many studies (Kaplan and Mason, 2008; Xiang *et al*., 2013). Other risk factors for resistant pneumococcal carriage include young age, attendance at day care centers and compromised immune system due to age and infections such as HIV in some populations (Henderson *et al*., 2016; Jones *et al*., 2018).The cross-resistance observed in this study is consistent with the principal mechanism of penicillin resistance in *S. pneumoniae*, in which the alterations in penicillin-binding proteins (PBPs) greatly decrease the affinity for almost all β-lactam antibiotics, including the third generation cephalosporin, ceftriaxone as reported by Xiang *et al*.(2013).In the contrary, Nigerian studies reported a good susceptibility rate to these antibiotics by *S. pneumoniae* (Habib *et al*., 2003; Adeleye *et al*., 2008; Obaro *et al*., 2011).

It was also observed that the organism was resistant to the macrolide, erythromycin.Macrolides have long been important in treating pneumonia, because of their excellent activity against pneumococci. This activity has been eroded in the past few years by the proliferation of macrolide-resistant strains. The prevalence of these resistance strains continues to increase in many parts of the world (Wu *et al*., 2013). High resistance to erythromycin were also reported by other researchers in Nigeria (Iwalokun *et al*., 2012; Akanbi *et al*., 2018).The high macrolide resistance may be traceable to the inappropriate use of macrolides, which are frequently associated with imprudent practice, in which antibiotics are routinely given to patients without known pathogens. Contrary to our findings, Onipede *etal*. (2019) reported 100% susceptibility of invasive pneumococcal isolates from patients to erythromycin which is also similar to findings by Adeleye *etal*. (2018) among sputum isolates from HIV-infected pediatrics and adult patients.

Susceptibility of *S. pneumoniae* to augmentin (which is actually a combination of amoxillin and clavulanate) was observed in this investigation. This is a typical case of synergy in drugs combination. Augmentin is not commonly used in Nigeria in the treatment of pneumococcal infection due to the cost of the drug. This drug is not affordable by low income earners (Iroha *et al*., 2012). This may be responsible for the sensitivity of*S. pneumoniae* to itas observed in this study.

Chloramphenicol is an old antibiotic that has been so much abused in the past, as a first-line treatment for typhoid fever in most developing countries (Iwalokun *et al*., 2012). However, with the appearance of cheaper generic forms of quinolones, and appearance of chloramphenicol-resistant *Salmonellatyphi*, prescribers have moved away from chloramphenicol and this relief of pressure on the drug, may be the reason for the good performance of chloramphenicol in this study (Iliyasu *et al*., 2015).This corroborate with findings by other workers in Nigeria (Adeleye *et al*., 2018; Akanbi *et al*., 2018).

Furthermore, it was also noted that the organism was sensitive to ciprofloxacin and levofloxacin but resistant to norfloxacin. Varying rates of resistance to quinolones (even same generation) by *S. pneumoniae* have been reported (Agwu *et al*., 2014). Therefore, the usual practice in some health establishments where susceptibility test is carried out on one quinolone and another is used for treatment either due to cost constraints or availability should be discontinued.

Resistance to antibiotics of at least 3 different groups has been defined as multiple drug resistance (Kandakai-Olukemi and Dido, 2009). Even though the multiple antibiotic resistance was not very high in this study when compared with those in some hospitals in other countries (Ma*et al*., 2013), the test organism had 46.66% resistance to the 15 antibiotics used. The relatively high resistance of our isolate to penicillin derivatives and other antibiotics limits the choice of antimicrobials that can be used for the treatment of pneumococcal infections.Thus, it is highly advised not to select macrolides as the first-line drug for treating *S. pneumoniae* infection.Statistical analysis revealed that there was no significant difference amongst the zones of inhibition at ($P \leq 0.05$).

CONCLUSION

In summary, the current study provided updated information and changing trends in the antimicrobial resistance pattern of *S. pneumoniae*. The data showed high resistance to penicillin derivatives, aminoglycosides, macrolide and an increasing multidrug resistance of the pneumococcal pathogen. Given the high resistance and its clinical impact, continuous surveillance of pneumococcal epidemiology is strongly recommended. The present study also has limitations. For instance, all the isolates were from one hospital and the situation of antibiotics therapy administered to patients was unclear, leading to bias and affecting the results of this study. In addition, only one identified *S. pneumoniae*isolate was used in the susceptibility study, and not the entire isolates. Therefore, isolates from more patients across many wards in the hospital should be investigated.

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