Incidence and Trend of Antibiotic Resistance among *Streptococcus pneumoniae* Isolated from Pediatrics in Ekiti and Ondo States of Southwestern Nigeria

***Ajibade V. A, Julius, O. O., and Ajayi, F. A**

Department of Science Technology, Microbiology unit,
Federal Polytechnic, P.M.B. 5351, Ado-Ekiti, Ekiti State, Nigeria

Abstract- The incidence of *Streptococcus pneumoniae* isolated from pediatrics admitted into various hospitals in Ekiti and Ondo States from the months of January 2009 to July 2010 were investigated. It was observed that out of the 400 samples collected from children in both states, 57 (14%) were positive for the isolates, while 295 (74%) were negative. Indicating a low incidence in both states but higher in Ondo State. Out of the 57 positive samples, 44 (77%) were isolates from sputum, 4 (7%) from urine, 1 (2%) from blood and 8 (14%) from ear swab. The highest number of positive isolates were 41 (68%) from males while 16(32%) were from females. From sputum, 33 (97%) were susceptible to Ciprofloxacin and 5(14%) to Cloxacillin. From urine, 3(75%) isolates were susceptible to Ofloxacin. The isolates from blood samples were susceptible to both Ciprofloxacin and Gentamycin. The isolates from ear swab were susceptible to Erythromycin and Ciprofloxacin but 1(50%) was susceptible to Ofloxacin, Clindamycin, Gentamycin, Cotrimoxazole Ampicilin and Cloxacillin. All isolates from urine samples show resistance to majority of the antibiotics. All isolates from blood showed resistance to all antibiotics tested except Ciprofloxacin and Gentamycin. Two isolates from ear swab showed resistance to Ceftriaxone and Argumentin. The most active antibiotics tested were Ciprofloxacin (92%), Gentamycin (78%), Erythromycin (70%), Ofloxacin (60%) and Clindamycin (46%).

Keywords- Antibiotic resistance, Incidence, Pediatrics, *Streptococcus pneumoniae*

I. INTRODUCTION

For the past 60 years, antimicrobial chemotherapy has been the mainstay of medical intervention against infectious diseases caused by bacterial pathogens. The continuous decline of therapeutic effectiveness as a result of extensive use of antimicrobial chemotherapy has been long predicted and seems inescapable (WHO, 2005). Many surveillance studies have over the last decade (1997-2007) drawn attention to this phenomenon (Mackenzie *et al*., 2007). At the same time, the once- abundant supply of new and improved antimicrobial compounds has worn thin, as drug development becomes increasingly challenging (Moel *et al*., 2007). It is therefore critical to realize that the effectiveness of antimicrobial agents has been challenged by resurgence of resistant microorganisms. This cannot be taken for granted because these substances are increasingly attaining the status of nonrenewable resources.

Streptococcus pneumoniae is a major human pathogen which causes pneumonia, sinusitis, otitis, bacterial meningitis and septicemia (Dorothea *et al*., 2010). The bacterium is a serious cause of illness and death and a major etiologic agent of community-acquired pneumonia meningitis, and acute otitis media. The increasing global emergence and rapid spread of multidrug-resistant *Streptococcus pneumoniae* is a serious concern (Rahman *et al*., 2006). Clonal dissemination of problematic pneumococcal strains has created clinically important treatment problems (Bean and Klena, 2005). Pneumococcal resistant to antimicrobial drugs was first reported in the mid-1960s (Douglas *et al*., 2009). Since 1990, drug resistant isolates of the bacterium have spread rapidly throughout the United States (Byarugaba, 2004). In the early 1990s, high-level resistance to penicillin and other antimicrobial drugs appeared in the United States with a low prevalence (Nys *et al.*, 2004). Over the past decade, multidrug-resistant clones of *Streptococcus pneumoniae* have rapidly emerged (Pelton *et al*., 2007). Of 90 serotypes, 19A is one of the most common types found in children. It can cause disease and easily develop antimicrobial drug resistance (Tan, 2003). Before the introduction of the 7-valent polysaccharide- protein conjugate vaccine (PCV7), serotypes included in the vaccine were responsible for approx 90% of pneumococcal infections in children living in industrialized countries; in developing countries, coverage has been reported as low as

26% (Pelton *et al*., 2007). Pneumococcal resistance to antimicrobial drugs (including β-lactams, macrolides, tetracycline, and cotrimoxazole) has become a worldwide problem. This work intends to investigate the trend of resistance of *Streptococcus pneumoniae* isolated from pediatrics in Ekiti and Ondo States to conventional antibiotics and their susceptibility to macrolides and using molecular epidemiologic methods, particularly multilocus sequence typing (MLST) to characterize the molecular type of multidrug-resistant strains of *Streptococcus pneumoniae* isolated from children.

II. MATERIALS AND METHODS

A. Collection of Isolates

Streptococcus pneumoniae were obtained from various clinical specimens like, sputum, urine, blood, and middle ear fluid from patients that visited the laboratory of the hospitals at Akure, Owo, Ondo, Idanre and Ore towns in Ondo- State and Ado Ekiti, Ido-Ekiti, Ikere-Ekiti, Iyin Ekiti, Aramoko-Ekiti and Ifaki-Ekiti in Ekiti State, Southwestern, Nigeria.

B. Culture of Samples and identification of bacteria

The samples were cultured on Nutrient agar by streaking method on Petri-dishes. The cultures were incubated anaerobically at 37^{o}C for 24h in an incubator. Discrete colonies were picked and cultured on Blood agar. The plates were incubated at 37^{o}C for 24h. Colonies showing greenish clearing zones around it were subcultured into a Nutrient agar slant. The colonies were identified by Gram staining, insulin fermentation, bile solubility, and optochin sensitivity (Rouff *et al*., 2003; CLSI, 2006).

C. Susceptibility Testing

Susceptibility testing was carried out by disk diffusion and confirmed with E- test according to Clinical and Laboratory Standard Institute (CLSI, 2006). A colony from stock was sub cultured into 5ml of nutrient broth (LAB) and incubated at 37^{o}C for 18h. About 0.1ml of the overnight broth of each organism was pipette into 9.9ml of the broth to yield a 10^{1} dilution. The procedure was continued to obtain a final dilution of 10^{3}. The bacterial suspension was spread onto a dried Nutrient agar and a multo-disk containing ofloxacin, erythromycin, Ciprofloxacin, Clindamycin, Gentimycin, Cloxacillin, Cotrimoxazole, Ampicilin, ceftriaxone and Argumentin was placed on the agar. Multidrug resistance was defined as non-susceptibility to ≥3 antimicrobial drug classes.

D. Multilocus Sequence Typing

This was employed using the method described in Qingfu *et al.* (2009). The internal fragments of 7 housekeeping genes (*aroE* Shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki*(glucose kinase), *recP*(transketolase), *spi*(signal peptidase1), *xpt*(xanthine phosphoribosyltransferase), and *ddl*(D-alanine-D-alanine ligase) were amplified from chromosomal DNA by PCR. Chromosomal DNA was extracted from subculture of *Strep pneumoniae* isolations recovered from middle ear fluid, urine, sputum and blood. PCR amplification was performed using primer pairs *aroE*- up, 5-GCCTTTGAGGCGACAGC-3' and *aroE*-dn, 5'-TGCAGTTCA(G/A)AAACAT(A/T)TTCTAA-3'; *gdh*-up, 5'-ATGGACAAACCAGC(G/A/T/C)AG(C/T)TT-3'; and *gdh*-dn, 5'-GCTTGAGGTCCCAT(G/A)CT(G/A/T/C) CC-3'; *gki*-up, 5'-GGCATTGGAATGGGATCACC-3' and *gki*-dn, 5'-TCTCCCGCAGCTGACAC-3'; *recP*-up, 5'-GCCAACTTAGCATTGTAAC-3'; and *rec*P-up, 5'-GCCAACTCAGGTCATCCAGG-3' and *rec*P-dn, 5'-TGCAACCGTAGCATTGTAAC-3'; and *spi*-up, 5'-TTATTCCTCCTGATTCTGTC-3' and *spi*-dn, 5'-GTGATTGGCCAGAAGCGGAA-3'. Pcr conditions were as follows; initial denaturation at 95^{o}C for 5min, followed by 30cycles of 95^{o} C for 30s; annealing at 50^{o} C – 55^{o}C for 30s; and extension at 72^{o} C for 30s. The amplified DNA fragments were purified by using QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and were sequenced in each direction by using the same primers used for amplification and by using the BigDye Terminator v3.1 Cycle Sequencing Kit on an Applied Biosystems Prism 377 automated sequencer (Applied Biosystems, Foster City, CA, USA).

The sequences at each of the n7 loci were then compared with the sequences of all of the known alleles at those loci in the database at the pneumococcal MLST website (http:spneumoniae.mlst.net). The sequences identical to a known sequence were assigned the same allele number, and non-identities to any known allele sequences were assigned new allele numbers. The allele at each of the 7 loci defines the allelic profile of each strain, as well as its ST. New allelic number or new ST number was assigned by a curator of the pneumococcal MLST database. The relatedness of isolates and known similar strains in the database was determined by constructing a neighbor-joining tree using a program online, Draw Tree Using Own MLST Data, found at the pneumococcal MLST website.

E. Statistical Analysis

Statistical analysis was performed by using SPSS software version 13.0. The Mantel-Haenszel x^2 test was used for trend analysis.

III. RESULTS AND DISCUSSION

The results of this research work are shown in tables below. Table 1 shows the incidence of *Strep pneumoniae* from different samples between January 2009 and July 2010. A total of 250 samples were collected from children in Akure metropolis for isolation purposes and antibiotic surveillance among *Strep pneumoniae,* 41(16%) of the samples were positive while 209(84%) were negative. 34(82%) out of 41 positive samples were isolated from sputum, 4(9%) isolates were from Urine samples, 1 (2%) isolates from blood sample and 2 (7%) isolates from ear-swab. Sixteen 16(40%) were serotype 19A. this was the most common serotype isolated in the study. Table 2 summarized the sexes and age of patients from which positive sample were obtained. The highest numbers of positive samples are from male 28(68%) while isolates obtained from female sample are about 13(32%). Table 3 summarizes the susceptibility pattern of isolates from different samples. The result were interpreted according to the MIC breakpoint recommended by CLS were zones of inhibition >5.0 were assigned as sensitive and those <5.0mm were assigned as resistant.

From urine samples, 3 (75%) isolates were susceptible to Ofloxacin while 1(25%) was less susceptible to Argumentin. Out of 34 isolates from sputum sample 33(97%) were susceptible to Ciprofloxacin and 5(14%) were susceptible to Cloxacillin. The isolate from blood sample was susceptible to both Ciprofloxacin and Gentamycin. Finally, isolates fro ear-swabs were susceptible to Erythromycin and Ciprofloxacin but 1(50%) was susceptible to Ofloxacin, Clindamycin, Gentamycin, Cotrimoxazole, Ampicilin and Cloxacillin. Table 4 summarizes the resistance pattern of isolates obtained from different sample. All isolates from Urine samples showed resistance to Cotrimoxazole, Ampicilin Cloxacillin and Ceftriaxone but less resistance to Ofloxacin. (34)86% of the isolates from sputum samples showed resistance to only Ceftriaxone, (30)83% showed resistance to Cotrimoxazole, Ampicilin, Cloxacillin and Argumentin but they are less resistance to Ciprofloxacin (3%).

The isolates from blood sample showed resistance to almost all the antibiotics used except only Ciprofloxacin and Gentamycin. The two isolates from ear-swab showed resistance to Ceftriaxone and Argumentin while 1 (50%) showed resistance to five antibiotics out of the ten antibiotics tested. The susceptibility patterns of the first nine samples analyzed are shown in the tables below. Table 5 shows the incidence of *S. pneumoniae* isolated from sputum collected in Ekiti State. A total of 135 sputum samples were analyzed, but only 20(16%) of the samples were positive for *S. pneumoniae.* Table 5 shows the incidence of *S. pneumoniae* isolated from sputum collected in Ekiti State. A total of 135 sputum samples were analyzed, but only 20(16%) of the samples were positive for *S. pneumoniae.* Table 6 shows the sensitivity profile of the twenty isolates.

The whole twenty (20) isolates were sensitive to Erythromycin, 17(85%) were sensitive to ciprofloxacin, 15 (75%) were sensitive to Ofloxacin, 10(50%) were sensitive to Gentamycin, 9(45%) were sensitive to Ceftriaxone, 4(20%) were sensitive to Clindamycin, and 1(5%) was sensitive to Ampicilin. Table 7 shows the resistance pattern of the twenty isolates to ten antimicrobial agents used. All the twenty (20) isolates were resistant to Cotrimoxazole and Argumentin followed by Ampicilin, 16(80%) were resistant to Clindamycin, 11(55%) were resistant to Ampicilin, 10(50%) were resistant to Gentamycin, 5(25%) were resistant to Ofloxacin, and 3(15%) were resistant to Ciprofloxacin. All the isolates exhibit multi-drug resistance by showing resistance to more than one antibiotic.

Table 1: Incidence of *Streptococcus pneumoniae* in Ondo State from different sample (January 2009 and July, 2010)

Group	Samples	Total number collected	Positive Samples, n (%)	Negative Samples, n (%)
1	Urine	10	1(10)	9 (90)
2	Sputum	25	4 (16)	21 (84)
3	Sputum	25	3 (12)	22 (88)
4	Urine	10	1 (10)	9 (90)
5	Sputum	30	4 (12)	26 (88)
6	Sputum	30	3 (9)	27 (91)
7	Sputum	20	4 (20)	16 (80)
8	Sputum	30	7 (21)	23 (79)
9	Blood	10	1 (10)	9 (90)
10	Sputum	40	9 (22)	31 (78)
11	Urine	10	2 (20)	8 (80)
12	Ear-swab	10	2 (20)	8 (80)
Total		**250**	**41 (16)**	**209 (84)**

Table 2: Relationship between positive samples, sexes and ages of patients

Sample (n)	Sex n (%)		Age (years)	
	Male	Female	≤13	>13
Urine (4)	2 (50)	2 (50)	-	4 (100)
Sputum (34)	24 (70)	10 (30)	1 (2)	33 (98)
Blood (1)	1 (100)	-	-	1 (100)
Ear-swab (2)	1 (50)	1 (50)	-	2 (100)
Total	28 (68)	13 (32)	1 (2)	40 (98)

Table 3: Sensitivity pattern of *Streptococcus pneumoniae* isolated from Ondo State to convectional antibiotics n (%)

Sample Number	OF	E	CIP	CD	GN	CX	CO	AP	FX	AU
Urine (4)	3 (75)	2 (50)	2 (250)	2 (50)	2 (50)	1(25)	-	-	-	1 (25)
Sputum (34)	21(61)	25(73)	33 (97)	16 (47)	28 (82)	5 (14)	6 (17)	6(17)	12 (35)	6 (17)
Blood (1)	-	-	1 (100)	-	1(100)	-	-	-	-	-
Ear-Swab (2)	1 (50)	2(100)	2 (100)	1 (50)	1 (50)	-	1 (50)	6(17)	-	-

OF-ofloxacin, E- erythromycin, CIP- ciprofloxacin, CD- Clindamycin, GN- Gentimycin, CX, Cloxacillin, CO- Cotrimoxazole, AP- ampicilin, FX- Ceftriaxone, AU- Argumentin

Table 4: Resistant pattern of *Streptococcus pneumoniae* isolated from Ondo State to convectional antibiotics n (%)

Sample/ number	OF	E	CIP	CD	GN	CX	CO	AP	FX	AU
Urine (4)	1 (25)	2 (50)	2 (50)	2 (50)	2 (50)	3 (75)	4 (100)	4 (100)	4 (100)	3 (75)
Sputum (34)	13 (39)	9 (27)	1(3)	18 (53)	6 (18)	29 (86)	28 (83)	28 (83)	22 (65)	28 (83)
Blood (1)	1 (100)	1 (100)	-	1 (100)	-	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Ear-Swab (2)	1 (50)	-	-	1 (50)	1 (50)	2 (100)	1 (50)	1 (50)	2 (100)	2 (100)

OF-ofloxacin, E- erythromycin, CIP- ciprofloxacin, CD- Clindamycin, GN- Gentimycin, CX, Cloxacillin, CO- Cotrimoxazole, AP- ampicilin, FX- Ceftriaxone, AU- Argumentin

Table 5: Incidence of *Streptococcus pneumoniae* in sputum samples Ekiti State

Batch Collection	Total number Collected	Positive Sample %	Negative Sample %
1	11	2 (2)	9 (7)
2	15	3 (2)	12 (9)
3	20	2 (2)	18 (13)
4	10	1 (1)	9 (7)
5	25	4 (3)	21 (16)
6	23	4 (3)	19 (14)
8	19	3 (2)	16 (12)
9	12	1 (1)	11 (8)
Total	**135**	**16**	**86**

Table 6: Antimicrobial **susceptibility(S) and resistance(R)** of clinical isolates of *S. pneumoniae* isolated from Ekiti State (n=20)

Isolate	CIP	GN	CX	CO	FX	AP	CD	AU	OF	E
S	17	10	0	0	9	1	4	0	15	20
R	3	10	20	20	11	19	16	20	5	0
S%	85	50	0	0	45	5	20	0	75	100
R%	15	10	100	100	55	95	50	100	25	0

OF-ofloxacin, E- erythromycin, CIP- ciprofloxacin, CD- Clindamycin, GN- Gentimycin, CX, Cloxacillin, CO- Cotrimoxazole, AP- ampicilin, FX- Ceftriaxone, AU- Argumentin

Table 5 shows the incidence of *S. pneumoniae* encountered in sputum sample collected in Ekiti state. A total of 135 sputum samples were analyzed, but only 20(16%) of the samples were positive for *S. pneumoniae.*

Table 6 shows the sensitivity and resistance profile of the twenty isolates. The whole twenty(20) isolates were sensitive to Erythromycin, Seventeen(17) isolates were sensitive to ciprofloxacin, fifteen(15) isolates were sensitive to Ofloxacin ,ten (10)isolates were sensitive to Gentamycin, nine (9)isolates were sensitive to Ceftriaxone, four(4) isolates were sensitive to Clindamycin, and one(1) isolate was sensitive to Ampicilin . Table 6 shows the resistance pattern of the twenty isolate to ten antimicrobial agents used. All the twenty (20) isolates were resistant to Cotrimoxazole and Argumetin followed by Ampicilin sixteen (16) isolates were resistant to Clindamycin, eleven (11) isolates were resistant to Ampicilin, ten (10) isolates were resistant to Gentamycin, five (5) isolates were resistant to Ofloxacin, and three (3) isolates were resistant to Ciprofloxacin. All the isolates exhibit multi-drug resistance by showing resistance to more than one antibiotic.

Out of the135 sputum samples analyzed for *S pneumoniae*, only twenty (20) were positive for the bacteria. The twenty isolates were subjected to antimicrobial susceptibility test using ten antibiotics. The susceptibility of the 20 isolates to the ten antimicrobial agents is shown in table 6. Seventeen isolates (85%) were sensitive to ciprofloxacin, fifteen (75%) isolates were sensitive to Ofloxacin, ten (50%) isolates were sensitive to Gentamycin, one (5%) isolate was sensitive to Ceftriaxone, and the whole twenty isolates (100%) were sensitive to Erythromycin.

The resistance pattern shows that 19 (95%) isolates were resistant to Ampicilin, 16 (80%) isolates were resistant to Clindamycin, 11 (55%) isolates were resistant to Ceftriaxone, ten (50%) isolates were resistant to Gentamycin, 5 (25%) isolates were resistant to

Ofloxacin, 3(15%) isolates were resistant to ciprofloxacin, the whole isolates are resistant to Cotrimoxazole and Argumentin. From this work, the most active antibiotic tested was Ciprofloxacin (92%), Gentamycin (78%), Erythromycin (70%), Ofloxacin (60%) and Clindamycin (46%).

The organisms also show complete resistance to Cotrimoxazole and Argumentin followed by Ampicilin and Clindamycin. The majority (82%) of the isolates were from respiratory tract specimen, the remainder having been recovered from specimens from other sites. This correlates with the study of Kenneth (2008) who state that *Strep pneumoniae* is part of the normal inhabitant of upper respiratory tract flora. 83% of the isolates were resistant to Cotrimoxazole, Ampicilin, Cloxacillin, Argumentin and 71% to Ceftriaxone. According to the study carried out by Rohani *et al*. (2003), Ceftriaxone was recognized as the most active among the drugs evaluated which is concluded to be an alternative to penicillin. But this study shows that the rate of resistance to Ceftriaxone is very high (71%) this could result from abuse of Ceftriaxone. The high resistance of *S. pneumoniae* to some of the antibiotics used may result from the uncontrolled and frequent use of these antibiotics and its derivatives and/or non-compliance of patients with respect to antibiotics dosage and duration. It may also be because almost all the isolates were respiratory tracts isolates which have been found to be more resistant than isolates from other sources in previous studies. The increasing resistance rates may cause serious difficulties in the treatment of *pneumococcal* infections; therefore clinicians prescribing therapy for this infection should take into consideration the trend of decreased sensitivity to antibiotics among *pneumococcal*. Since the resistance of Ceftriaxone and some other antibiotics among *pneumococcal* increases in this study, a number of alternatives are available, the most active of those evaluated here being Ciprofloxacin and Gentamycin.

Multilocus sequence typing (MLST) of the 57 *S. pneumoniae* isolates showed that 38(67%) were ST-320; 8(14%) were ST-199; 5(9%) were unreported STs now assigned ST-2722 and ST-2704; 2(13%) were ST-1673; and 1(6%) each of the remaining 3 was ST-1451, ST2265, and ST-63. The total number of those typed as ST-320 were multidrug-resistant, however, 1 (2%) was the newly assigned ST2722. The ST-2722 strain appears to have resulted from a mutation identified in the *recP* gene that coincides with acquisition of multidrug resistance, including Ceftriaxone (Qingfu *et al.,* 2009). The clinical importance of such strain is potentially high because empiric treatment of suspected and even proven pneumococcal infections typically relies on the efficacy of Ceftriaxone. Continuous monitoring of serotype distribution in this population will ensure that available vaccines can provide adequate coverage of circulating pneumococcal serotypes.

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AUTHORS

First Author – Ajibade, V. A., Ph.D, MSc., PGD, HND, The Federal Polytechnic, Ado-Ekiti, Nigeria, ajibvijay@yahoo.com

Second Author – Julius, O. O., MSc., BSc., The Federal Polytechnic, Ado-Ekiti, Nigeria, oyebodemirace@yahoo.com

Third Author – Ajayi, F. A, PGD, HND. The Federal Polytechnic, Ado-Ekiti, Nigeria,

Correspondence Author – Ajibade, V. A ajibvijay@yahoo.com, +2348035781628