International Journal of Medicine and Medical Sciences Vol. 4(8), pp.160-170, October 2012
Available online at http://www.academicjournals.org/IJMMS
DOI: 10.5897/IJMMS12.054
ISSN 2006-9723 ©2012 Academic Journals

Full Length Research paper

A retrospective study of clinical *Streptococcus pneumoniae* isolates from four health facilities in South-West Nigeria

Iwalokun Bamidele A.[1,3]*, Fowora, M.[2], Akinloye Olubukola[3], Oluwadun Afolabi[3], Antonio M.[4] and Adegbola, R. A.[4]

[1]Biochemistry and Nutrition Division, Nigerian Institute of Medical Research, P. M. B. 2013, Yaba-Lagos, Nigeria.
[2]Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research, P. M. B. 2013, Yaba-Lagos, Nigeria.
[3]Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria.
[4]Medical Research Council (MRC). Atlantic Boulevard, Fajara, Gambia.

Accepted 29 June, 2012

Nigeria is currently one of the highest pneumococcal disease burdened countries not implementing routine pneumococcal conjugate vaccine (PCV) immunization and having limited clino-biological data on *Streptococcus pneumoniae*. This retrospective study provides phenotypic and genotypic data on 15 isolates of *S. pneumoniae* recovered from clinical samples provided by 75 bacteremia, asthma, *pneumonia*, otitis media, meningitis, severe malaria and sickle cell anaemia (SCA) patients, attending health facilities within the south-West region of the country. The recovered *S. pneumoniae* isolates were serotyped and had their antibiotic susceptibilities determined by disk diffusion and MIC assays. They were further analyzed for disparity by SDS-PAGE and RAPD analysis coupled with genotyping for ply and lyt genes to query virulence. Empirical antibiotic prescription and demographic data were also extracted from the patients' medical records with consent. The 15 recovered *S pneumoniae* isolates belonged to 5 distinct serotypes: 19F (n = 6), 5 (n = 3) and 2 each of 6B, 9V and 23F. More isolates were recovered from children than adults and from invasive diseases than non-invasive ones. However, serotype 9V isolates (adults only) were distinctively invasive and genotyping revealed some levels of clonal diversity and virulence among the multi-drug resistant strains. All the strains were within the vaccine coverage of PCV-13.

Key words: *Streptococcus pneumoniae*, serotypes, antibiotic susceptibility, random amplified polymorphic DNA (RAPD), pneumococcal conjugate vaccine coverage, Nigeria.

INTRODUCTION

Streptococcus pneumoniae remains a leading cause of morbidity and mortality from meningitis, bacteremia and pneumonia in Nigerian children, aged-adults and the immuno-compromised such as sickle cell anemia and HIV patients (Akuse, 1996; Adeleye et al., 2008; Obaro, 2009). Currently, pneumonia, which is one of the diseases caused by *S. pneumoniae*, kills 200,000 Nigerian children below 5 years annually (Onche, 2009). This makes Nigeria a high pneumonia burdened country where children are 17 – 400 times more likely to die from pneumonia than a child living in the US 'World Pneumonia Day Media Report, 2010'. Recent studies in Nigeria have also implicated *S. pneumoniae* as 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 (Falade et al., 2009; Obaro et al., 2011).

Like in other endemic countries of the world, where three-quarter of global pneumonia deaths occur, risk factors that have been identified for invasive pneumococcal diseases (IPD) due to *S. pneumoniae* in Nigeria include air pollution, overcrowding, naso-pharyngeal carriage and high level transmission of

*Corresponding author. E-mail: bamwal@yahoo.com. Tel: +234-1-8023357646.

the pathogen as well as the presence of co-morbidities such as HIV/AIDS and sickle cell anemia (Akuse, 1996; Akinsete et al., 1998; Media report, 2011; Adetifa et al., 2012). Deaths resulting from *S. pneumoniae* infections have been attributed to its capsular polysaccharide cell wall that gives rise to over 90 serotypes, and protein factors such as autolysin (*lytA*) and pneumolysin (*ply*) that are involved in invasion, disease progression, and protection from host mediated opsonization and phagocytic killing (Appelbaum, 1992). Furthermore, the country is also plagued by an inadequate health system with sub-optimal vaccine coverage that is presently at 72% for non-pneumococcal vaccines (WHO-UNICEF, 2010), disease surveillance and health system research in the last 20 years (Uneke et al., 2010).

Meanwhile in pneumonia burdened countries, two-third of annual deaths could be averted if 90% of children had access to simple and effective pneumococcal vaccine (Media Report, 2011). Therefore, to address the pneumococcal problem in response to the global call for action against pneumonia, Nigeria has planned to integrate pneumococcal conjugate vaccine (PCV) into her national immunization program (NIP) in 2013 and promote etiology research for improved case management and preventive measures. Expectations of benefits from PCV usage is based on the lessons learned from the presently implementing countries worldwide in which life-year, quality adjusted life year and reduction in colonization, transmission and burden of invasive pneumococcal diseases (IPDs) in the post PCV era have been reported (Falade et al., 2009; Adetifa et al., 2012).

In the meantime, serotype data of circulating *S. pneumoniae* strains in the country are needed for making PCV procurement decision, evaluating serotype-specific disease burden and establishing baseline indicators to monitor and evaluate for outcomes and impact of prevention and surveillance programmes in the post PCV era. Information on drug response and clonal diversity of the circulating strains are also needed for better understanding of *S. pneumoniae* epidemiology and development of control program and policy in Nigeria. It is in this context that the present study was conducted with the aim of having a snapshot on the gene diversity by randomly amplified polymorphic DNA (RAPD) and whole cell protein profiling of *S. pneumoniae* strains form South-West, Nigeria. Sensitivity to anti-pneumococcal antibiotics by these isolates was also evaluated.

METHODOLOGY

Study design

This was a retrospective study of 15 *S. pneumoniae* isolates recovered from clinical samples that were obtained from 75 patients aged 1 – 62 years at 4 health facilities in Lagos and Ibadan, Oyo State, between March to July, 2010 and June 2011. The health facilities attended by the patients in Lagos were Massey Street Children Hospital, Lagos University Teaching Hospital (LASUTH), and Onikan Health Centre, Lagos, while the cultured clinical samples from Ibadan came from patients attending University College Hospital (UCH). The presenting clinical conditions of the patients were severe malaria, sickle cell crisis associated pneumonia, bacteremia, otitis media, asthma, meningitis and chronic sinusitis. Pneumonia was diagnosed on the basis of chest radiological findings; positive Cerebrospinal fluid (CSF) culture results defined some meningitis cases, while bacteremia was based on positive blood culture results. These case definitions of patients included the use of clinical symptoms and case definition guidelines of Pneumococcal Vaccines Accelerated Development Plan (PneumoADIP) (Falade et al., 2009). Informed consent was obtained from the patients (adults) or their guardians for children before sample collection.

This study is ancillary to the respiratory pathogen surveillance study (Akinloye et al., 2011) approved by the Ethical Committee of the Oyo State Ministry of Health. The clinical samples submitted by the patients from whom *S. pneumoniae* strains were isolated were blood, cerebrospinal fluid (CSF), sputa (Bartlett grading complaint) and middle ear exudates according to their presenting clinical conditions.

Bacteriology and serological testing

Samples were cultured on 5% sheep blood agar (SBA) and chocolate agar plates by direct inoculation using a sterile loop. Blood samples (1 ml per patient) were cultured in trypticase soy broth (TSB) and Brain Heart Infusion broth (BHI) for 24 to 48 h for 7 days with sub-culturing on the agar plates on days 2, 3 and 7. The inoculated plates were incubated aerobically (5% sheep blood agar plates) and in a 5% CO_2 candle jar (chocolate agar plate) at 37°C for 24 to 28 h. The plates were examined thereafter for bacterial growth and positive plates were submitted to morphological evaluation by Gram staining and *S. pneumoniae* biochemical tests such as optochin sensitivity (IZD > 14 mm), 2% deoxycholate solubility and alpha haemolysis. To further confirmed the recovered *S. pneumoniae* isolates, API 20 Strep system (bioMerieux, France) according to manufacturer's directive was used. Serotyping was done using capsular and human factor sera based on antibody-coated latex agglutination assay (Denka, Seiken, Japan).

Antibiotic susceptibility testing (AST)

The isolates were screened for sensitivity to antibiotics by disc diffusion method on Mueller-Hinton agar supplemented with 5% defibrinated horse blood and nicotinamide adenine dinucleotide (20 ug/ml, Sigma, USA) using overnight culture from pure colonies of *S. pneumoniae* (on sheep blood agar plate) suspended in Mueller Hinton (MH) broth at 0.5 MacFarland standard density equivalent and standard antibiotic disks from Oxoid, UK, as follows: oxacillin, 1 µg; erythromycin, 15 µg; amoxicillin-clavulanate (AMC, 20/10 µg); tetracycline, 30 ug; trimethoprim/sulphamethoxazole (COT, 1.25/23.75 µg); chloramphenicol, 30 µg; cefotaxime, 30 µg; ceftriaxone, 30 µg; ciprofloxacin, 5 µg and laevofloxacin, 5 µg. After disk mounting, the inoculated plates were incubated at 35°C for 20 h under 5% CO_2 atmosphere. Plates were then examined for zones of inhibition with diameter produced measured in millimeters (mm) and interpreted as sensitive, intermediate resistance and resistance according to antibiotic breakpoint inhibitory zone diameter interpretation guidelines of the Clinical Laboratory Standard Institute (CLSI, 2006). Isolates with oxacillin zone sizes of $\geq$20 mm and $\leq$ 19 mm were interpreted as penicillin sensitive and resistant respectively, while those with COT zone sizes of $\geq$ 19 mm, 16 -18 mm and $\leq$ 15 mm were referred to as sensitive, intermediate resistant and resistant isolates. Erythromycin zone sizes of $\geq$ 23 mm, 14 - 22 mm and $\leq$ 13 mm were indicative of sensitive,

intermediate and resistant isolates, while chloramphenicol or tetracycline zone sizes of > 21 mm and < 20 mm were indicative of sensitive and resistant isolates.

Minimum inhibitory concentrations (MICs) for penicillin, amoxicillin-clavulanate, ceftriaxone and ciprofloxacin were determined by microbroth dilution method with results interpreted using antibiotic MIC breakpoints recommended by National Committee for Clinical Laboratory Standards (NCCLS, 1990) for *S. pneumoniae*. Briefly, penicillin MICs of $\leq$ 0.06, 0.12 to 1 and $\geq$ 2 µg/ml were indicative of sensitivity, intermediate resistance and resistance respectively, while for amoxicillin/clavulanate, MICs of $\leq$ 2, $\geq$ 4 and $\geq$ 8 µg/ml defined sensitive, intermediate resistance and resistance respectively. Ceftriaxone MICs of < 1 and $\geq$ 2 µg/ml defined sensitive and resistance isolates, while for ciprofloxacin, MICs of $\leq$ 1 and $\geq$ 4 µg/ml were definitions for sensitive and resistant isolates, respectively. In both disk diffusion and MIC determination assays, *S. pneumoniae* ATCC 49619 with penicillin resistance phenotype was used for quality control.

DNA preparation

A loopful of *S. pneumoniae* colony on SBA was suspended in 200 µL of 100 mM Tris-HCl buffer (pH 7.4), followed by the addition of proteinase k solution (0.5 mg/ml final concentration). The suspension was incubated at 37°C for 15 min, then boiled for 10 min and centrifuged at 8,000 rpm for 10 min after cooling. The resulting supernatant was then transferred to a fresh Eppendorf tube and 2.5 µL was used as the DNA template for polymerase chain reaction (PCR) and RAPD PCR assays.

Virulence gene detection

Two *S. pneumoniae* virulence genes: *lytA* and *ply* were amplified as 308 and 329 bp products by PCR using gene specific primers as described by Nagai et al. (2001) and Salo et al. (1995). The primer pair for *lytA* amplification was 5′-CAA CCG TAC AGA ATG AAG CGG-3′- F and 5′-TTA TTC GTG CAA TAC TCG TGC G-3′-R, while that of ply was 5′-ATT TCT GTA ACA GCT ACC AAC GA-3′-F and 5′-GAA TTC CCT GTC TTT TCA AAG TC-3′-R. The PCR cycling conditions for the amplification of both genes were as follows: 94°C for 2 min, followed by 25 cycles of 94°C for 10 s, 58°C for 15 s and 72°C for 60 s and a final extension step at 72°C for 5 min. The PCR products were recovered by electrophoresis on ethidium bromide (0.5 µg/ml) pre-stained 2% agarose gel.

RAPD PCR

An arbitrary primer 1254 ((5′-CCGCAGCCAA-3) from Biomers (Germany) was used for RAPD-PCR assay of *S. pneumoniae* DNA sample (100 ng) using a modified protocol of Duarte et al. (2005) in a 25-µL PCR reaction volume comprising dNTPs (200 µM each), primer (20 picomole), $MgCl_2$ (3.0 mM) and Taq polymerase (2.5 U) in 1X PCR buffer (20 mM Tris-HCl (pH 8.3) + 50 mM KCl). The PCR conditions were as follows: 2 cycles of 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min with a final extension step at 72°C for 10 min. RAPD-PCR products were resolved by electrophoresis on ethidium bromide (0.5 µg/ml) pre-stained 1.0% agarose gel using a 1 Kb DNA ladder (Fermentas) for size extrapolation. The RAPD profile of *S. pneumoniae* ATCC 49619 was used for quality control.

Analysis of cell protein profile by SDS-PAGE

Here bacterial cell pellet prepared by centrifuging (5,000 rpm for 10 min) *S. pneumoniae* BSA culture suspension in sterile water and decanting the supernatant was homogenized in 150 µL sample buffer (10% glycerol, 2% SDS, 5% β-mercaptoethanol in Tris-HCl buffer (pH 6.8). The resulting homogenate was then boiled at 80°C for 10 min, followed by centrifugation at 8,000 rpm for 10 min. Electrophoresis of the resulting protein sample (15 µL per well) was carried as described by Merquior et al. (1994) using 4 and 12.5% stacking and running gel, respectively. The protein profiles were compared by visual inspection.

Discriminatory power of RAPD-PCR technique

This was carried out using the discriminatory index described by Hunter and Caston (1988) as given by the equation: $D = 1 - [1/N(N-1)]\sum nj\,(nj-1)$, where D is the numerical index of discrimination, N is the total number of strains and nj is the number of strains pertaining to the jth type.

Data entry and analysis

Data obtained were entered and analyzed using SPSS 11.0 Statistical software. They were reported as number and percentages, median and range and mean (SD). Comparisons were done using chi-square, Fischer exact test, Kruskal-Wallis and Student's t-tests for percentages, median and mean values. P-values below 0.05 were considered to be significant.

RESULTS

Of the 75 patients aged 1 - 62 years who provided clinical samples for *S. pneumoniae* screening, 40 (53.3%) were children with 22 (55%) of these children aged $\leq$ 5 years. Other age groups accounted for 46.7% of the clinical samples cultured (P>0.05). Overall, the female patients were significantly (P<0.05) younger than the males, but this was not evident in the children age group (age 1 -12 years). Gender difference was also observed in the frequency of otitis media and bacteremia with more females than males and vice versa. However, gender disparity in the isolation of 15 *S. pneumoniae* strains was not significant (P>0.05) (Table 1). Results presented in Table 2 showed that the 15 *S. pneumoniae* strains isolated were recovered from cases of meningitis (n = 4), suppurative otitis media (n = 4), bacteremia (n = 2), sickle cell anemia associated pneumonia (n = 2), asthma (n = 2) and severe malaria (n = 1). Positive cultures were actually obtained from blood (n = 6), sputum (n = 2), ear swab (n = 3) and CSF (n = 2) samples respectively. The observed 40% isolation rate of the *S. pneumoniae* isolates from blood was also significant (P<0.05) when compared to other clinical samples. Furthermore, of the 15 recovered isolates, 3 came from Ibadan health facility, belonging to serotypes 5 (n = 1) and 19F (n =2) from a patient with severe malaria and two patients with otitis media (Table 2)

The results in Table 3 and Figures 1 and 2 provide the summary of serotype affiliation, clonal differentiation, antibiotic resistance profiles and virulence disposition of the 15 recovered *S. pneumoniae* strains. Isolates

Table 1. Demographic and clinicopathological characteristics of the patients.

Variable	Male	Female	Total	P
N (%)	40 (53.3)	35 (46.7)	75 (100)	> 0.05[a]
Age, years				
Range (Median)	1-62 (12.5)	4 -35 (12)	1- 62 (12)	> 0.05[b]
Mean (SEM)	19.2 (2.9)	13.3 (1.4)	16.4 (1.7)	< 0.05[c]
Age distribution, n(%)				
1-2	3 (4)	0 (0)	3 (4)	ND
3-5	10 (13.3)	9 (12)	19 (25.3)	> 0.05[a]
6-12	8 (10.7)	10 (13.3)	18 (24)	> 0.05[a]
13-19	3 (4)	8 (10.7)	11 (14.7)	< 0.05[d]
20-40	9 (12)	7 (9.3)	16 (21.3)	> 0.05[a]
41 and above	7 (9.3)	1 (1.3)	8 (10.7)	< 0.05[d]
Age group classification				
Children	21 (28)	19 (25.3)	40 (53.3)	> 0.05[a]
Adults	19 (25.3)	16 (21.4)	35 (46.7)	> 0.05[a]
Clinical condition				
S. pneumoniae, n(%)	9 (12)	6 (8)	15 (20)	> 0.05[a]
SCA-Pneumonia, n(%)	6 (8)	4 (5.3)	10 (13.3)	> 0.05[d]
Meningitis, n(%)	8 (10.7)	5 (6.7)	13 (17.4)	> 0.05[d]
Asthma, n (%)	8 (10.7)	11 (14.7)	19 (25.4)	> 0.05[a]
Supp. Otitis media, n(%)	1 (1.3)	7 (9.3)	8 (10.7)	< 0.05[d]
Bacteremia, n(%)	13 (17.4)	2 (2.6)	15 (20)	< 0.05[d]
Severe malaria, n(%)	4 (5.3)	6 (8)	10 (13.3)	> 0.05[d]

Data are reported as number or percentages, mean (SD) and median (range). [a]Chi-square test; [b]Kruskall-Wallis ; [c]Student's t-test;; [d]Fischer exact test. P-values < 0.05 are significant.

Table 2. Distribution of isolated *S. pneumoniae* strains by samples and disease conditions and health facilities.

Cases/Samples (N)	Blood	Sputum	Ear exudates	CSF	Total
Severe Malaria, SM (10)	**23F**	-	-	-	1
SCA- Pneumonia (10)	**5**, 23F	-	-	-	2
Bacteremia, Bac (8)	9V, 9V	-	-	-	2
Asthma, AS (19)	-	19F, 19F	-	-	2
Otitis media, OM (15)	-	-	6B, 19F, **19F, 19F**	-	4
Meningitis, M (13)	6B	0	0	5, 5 19F	4
Total (75)	6 (40)[a]	2 (13.3)	4 (26.7)	3 (20)	**15 (100)**

Data are numbers with overall percentages in parenthesis. Bolded serotypes are from Ibadan, while un-bolded serotypes are from Lagos health facilities. [a]P<0.05 compared to isolation rate from other clinical samples (Fischer exact test).

belonging to serotype 19F accounted for 40% (6 of 15 serotypes) of all serotypes recovered, followed by serotype 5 (3 of 15, 20%) and 2 each of serotypes 6B, 9V, 19F and 23F (Table 3).

RAPD and SDS-PAGE analyses revealed six and three distinct genotypes (1 – 6) and proteotypes (1 - 3) respectively, with most of 19F and 23F isolates belonging indistinguishably to RAPD types 1 and 2 and proteotypes A and B, respectively. Overall, these isolates were positive for *ply* and *lyt* genes, suggesting that they are clinically important. Disk diffusion assays revealed that all the isolates elicited resistance to penicillin and tetracycline, while resistance rates to other resisted antibiotics were chloramphenicol (66.7%), erythromycin

Table 3. Distribution of *S. pneumoniae* isolates by serotypes, RAPD types, virulence expression and antibiotic resistance.

Isolate	Age (year)	Case	RAPD type	Serotype	Protein profile	PEN	CHL	TET	ERY	TMP/SMX	AMC	CRO	CIP	LAEV	CTX	*lyt*	*ply*
SP_01	3	M	1	19F	A	R	R	R	S	S	S	S	S	S	S	+	+
SP_02	1	M	1	19F	A	R	S	R	S	S	S	S	S	S	S	+	+
SP_03	4	M	2	19F	A	R	R	R	S	S	S	S	S	S	S	+	+
SP_04	23	OM	3	5	A	R	R	R	S	R	S	S	S	S	S	+	+
SP_05	42	Bac	NT	9V	B	R	R	R	R	R	R	S	S	S	S	+	+
SP_06	7	AS	4	5	C	R	R	R	R	R	S	S	S	S	S	+	+
SP_07	9	SCA-P	4	6B	A	R	S	R	R	S	R	S	S	S	S	+	+
SP_08	14	OM	5	5	A	R	R	R	S	R	R	S	S	S	S	+	+
SP_09	12	OM	2	23F	A	R	S	R	R	S	S	S	S	S	S	+	+
SP_10	2	SM	1	23F	B	R	S	R	R	S	S	S	S	S	S	+	+
SP_11	5	AS	6	6B	A	R	R	R	R	S	S	R	S	S	S	+	+
SP_12	11	OM	1	19F	B	R	R	R	S	R	R	S	S	S	S	+	+
SP_13	62	Bac	NT	9V	A	R	R	R	R	R	R	S	S	S	S	+	+
SP_14	4	M	1	19F	C	R	S	R	S	S	S	S	S	S	S	+	+
SP_15	8	SCA-P	5	19F	B	R	R	R	S	S	S	S	S	S	S	+	+
					R (%)	100	66.7	100	46.7	40	33.3	6.7	0	0	0		

R% = Antibiotic resistance rate; SP_N (*Streptococcus pneumoniae* _strain no.)**;** NT **=** not typeable; PEN **=** penicillin; ERY **=** erythromycin; AMC **=** augmentin; CRO = ceftriaxone; CTX = cefotaxime; CIP **=** ciprofloxacin; LAEV **=** levofloxacin; R **=** resistance; S **=** sensitive; + **=** detected; *ply* = pneumolysin gene; *lyt* = autolysin gene; SCA-P = sickle cell anaemia –pneumonia; M = meningitis; AS = asthma; OM = Otitis media; SM = severe malaria;

(46.7%), trimethoprim/sulfamethoxazole (TMP/SMX) (40%), augmentin (33.3%) and ceftriaxone (6.7%). However, these isolates were sensitive to cefotaxime, ciprofloxacin and laevofloxacin (Table 3). Apart from the 23F and two of the 19F *S. pneumoniae* serotypes, other isolates were found to be multi-drug resistant and resulted in theproduction of 10 distinct serotype-dependent antibiotic resistance patterns with MAR indices of 0.2 - 0.6 with serotype 9V isolates as the most antibioticresistant strains. The latter was further confirmed by the MIC assays, which also revealed serotypes 23F and 6B strains to be more sensitive to the antibiotics tested and resistance to penicillin as intermediate for most isolates apart from serotype 9V and one of the three serotype 5 *S. pneumoniae* isolates recovered. Pneumococcal conjugate vaccine coverage rate analysis revealed coverage rates of 80 and 100% for PCV-7 and PCV-13, respectively (Table 4).

Microbroth dilution assays further confirmed that all the isolates except serotype 9V and 1 of the 3 serotype 5 strains had intermediate resistance to penicillin. Strains belonging to serotypes 23F and 6B were also observed to produce the lowest MICs for ceftriaxone, augmentin and ciprofloxacin, suggesting higher sensitivity when compared to other serotypes, while serotype 9V strains were the least sensitive to all the efficacious antibiotics tested (Table 5). However, isolates of serotypes 9V (adults only) and 5 (adult and children) were distinctively invasive and non-invasive in aetiology, while serotypes 6B and 19F were associated with SCA-associated pneumonia Table 6). rate to erythromycin and trimethoprim/sulfamethoxazole for invasive isolates over their non-invasive counterparts(Table 6). A total of 7 (46.6%) *S. pneumoniae* isolates were recovered from children $\leq$ 5 years and 72.3% from children ≤ 13 years. The disparity in isolation rates between these age groups and between ≤ 5 and 14 - 40 or 41 and above age categories was significant ($P<0.05$) (Table 7). Empirical prescription records from 41 of the 75 patients showed that most empirical prescriptions were monotherapies, highest for trimethoprim/sulfamethoxazole (26.8%) and lowest for ceftriaxone (9.8%). Other empirically prescribed antibiotics include penicillin (19.5%),

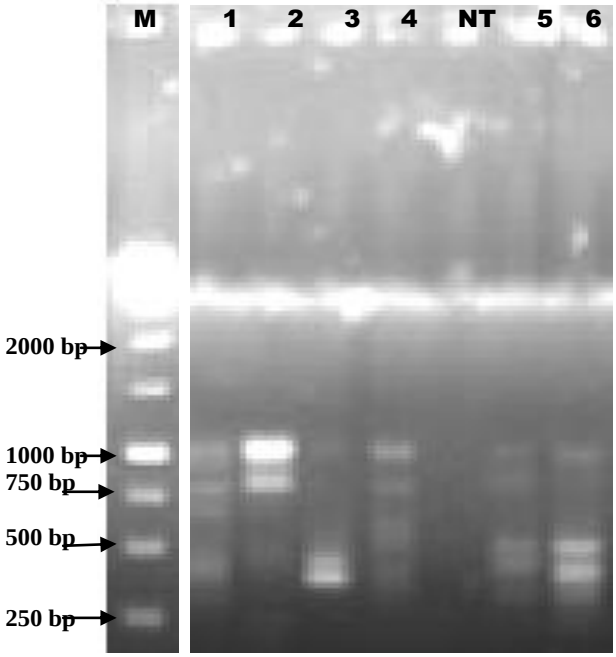

Figure 1. Agarose gel electrophoresis of distinct RAPD patterns elicited by the *S. pneumoniae* isolates.

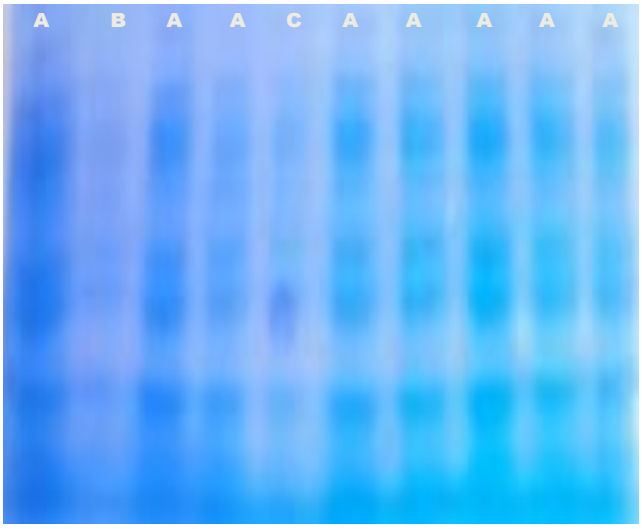

Figure 2. Whole cell protein profile by SDS-PACE of *Streptococcus pneumoniae* isolates screened. Wells with different letters represent distinct whole cell protein profiles.

augmentin (14.6%), ampicillin (17.1%) and ampicillin + gentamicin (2.4%) Further analysis revealed significant ($P < 0.05$) resistance (Table 8)

DISCUSSION

In this study, we recovered 15 isolates of *S. pneumoniae* of serotypes 5, 6B, 9V, 19F and 23F from 75 cultured samples as aetiologic agents of invasive (bacteremia, meningitis, SCA associated pneumonia) and non-invasive (otitis media, asthma) diseases with more isolates recovered from children than adults. These isolates showed resistance to 2 or more antibiotics, genetic diversity by RAPD analysis and exhibited PCV-7 and PCV-13 coverage rates of 80 and 100%, respectively. Our isolation rate of 20% (15 of 75 samples) was lower than 24.9 and 37% reported by Lagunju et al. (2008) and Ndip et al. (1995), but higher than 6.4% reported by Agwu et al. (2006) in Ekpoma, Nigeria, in 2006, 11% by Adeleye et al. (2008) in Lagos in 2008 and 7.6% by Obaro et al. (2011) in north-central, Nigeria in 2011. Apart from the sample size with ours being the lowest, the observed difference in *S. pneumonia* isolation rates may also be due to difference in study design.

Agwu et al. (2006) surveyed *S. pneumoniae* among *Mycobacterium tuberculosis* infected patients, Adeleye et al. (2008) conducted a cross-sectional study of *S. pneumoniae* in HIV/AIDS patients, Obaro et al. (2011), screened for *S. pneumoniae* only in children aged ≤ 5 years, while Lagunju et al. (2008) worked on children with meningitis only.

In the work of Ndip et al. (1995), the *S. pneumoniae* isolates were recovered from patients with otitis media and lower respiratory tract infections. In the year 2000, the national estimate of pneumococcal disease burden was reported to be 5% in children below 5 years, while 27% was reported for India (Chawla et al., 2010). Nevertheless, our results have revealed persistence of *S. pneumoniae* as etiological agents of bacteremia, sepsis, meningitis, otitis media and pneumonia in Nigeria. It also portends an increase in the trend of *S. pneumoniae* disease burden in the country with potential variations in prevalence/rates by geographical and disease settings. Furthermore, the observed higher isolation rate of *S. pneumoniae* in children than adults agrees with previous findings in Nigeria and other endemic countries of the world (Adetifa et al., 2012; Kim et al., 2010). This may be attributed to early colonization of *S. pneumoniae* in children, usually by week five (Antonio et al., 2008).

An important finding of this study is the recovery of serotype 9V *S. pneumoniae* isolates in Lagos that were not reported by previous authors. Falade et al. (2009) recovered serotypes 19F, 4 and 5 isolates from children with sepsis, meningitis and pneumonia in Ibadan, Nigeria, while Onyemelukwe and Greenwood (1982) reported serotypes 1, 2, 3 and 5 *S. pneumoniae* isolates as causes of invasive pneumococcal diseases (IPD) in Nigeria in 1982. An exception to this discrepancy was the recent report by Adetifa et al. (2012). The workers recovered serotype 9V *S. pneumoniae* as nasopharyngeal carriage isolates, accounting for ~2% of all isolates recovered. Meanwhile, serotype 9V *S. pneumoniae* isolates are commonly isolated IPD pathogens in patients from Europe and the USA (Tracey et al., 1999; Jenkins et al., 2008). This serotype has also

Table 4. Theoretical pneumococcal conjugate vaccine coverage rate, levels and serotype specific antibiotic resistance patterns among the isolated *S. pneumoniae* strains.

Serotype	n	@MAR index	Resistance pattern	^MDR phenotype (%)	Coverage rate, % PCV-7 PCV -13
5	3	0.4 0.5 0.5	PEN CHL TET TMP/SMX PEN CHL TET ERY TMP/SMX PEN CHL TET TMP/SMX AMC	100	80, 100
6B	2	0.4 0.5	PEN TET ERY AMC PEN CHL TET ERY CRO	100	
9V	2	0.6 0.6	PEN CHL TET ERY TMP/SMX AMC (2)	100	
19F	6	0.2 0.3 0.5	PEN TET (2) PEN CHL TET ERY (3) PEN CHL TET TMP/SMX AMC	66.7	
23F	2	0.3	PEN TET ERY (2)	100	

Figures in parentheses are number of resistance patterns. s@MAR index is calculated as the ratio of number of antibiotics resisted to total number of antibiotics tested; ^MDR phenotype is expressed as the percentage of total number of isolates eliciting resistance to 3 or more classes of antibiotics; Coverage rate is defined as the number of *S. pneumoniae* serotype recovered as a percentage of constituent serotypes in PCV-7 (4, 6B, 9V, 14. 18C, 19F, 23F) and PCV-13 (PCV-7 + 1, 2, 3, 5, 6C, 19A). n = Number of strains per serotype; PEN = penicillin; ERY = erythromycin; AMC = augmentin; TET = tetracycline; CHL = chloramphenicol; TMP/SMX = trimethoprim/sulfamethoxazole.

Table 5. Minimum inhibitory concentrations of tested antibiotics against the serotypes *Streptococcus pneumoniae*.

Serotype	Mean (SEM) MIC, µg/ml								
	Penicillin			Ceftriaxone		Augmentin		Ciprofloxacin	
	S	I	R	S	R	S	R	S	R
5	-	0.24 (0.07)	2.1 (0.07)	0.4 (0.05)	-	0.19 (0.05)	-	0.05 (0.007)	-
6B	-	0.18 (0.07)	-	0.16 (0.07)	-	0.09 (0.03)	-	0.02 (0.006)	-
9V	-	-	3.0 (0.7)	0.38 (0.1)	3.84	0.38 (0.1)	-	0.17 (0.07)	-
19F	-	0.21 (0.07)	-	0.18 (0.03)	-	0.19 (0.05)	-	0.08 (0.02)	-
23F	-	0.54 (0.1)	-	0.078 (0.02)	-	0.039 (0.01)	-	0.04 (0.002)	-

Table 6. Evaluation of antibiotic resistance between invasive and non-invasive serotypes of *Streptococcus pneumoniae* isolates recovered.

Antibiotic	Invasive isolates [n = 9:5 (3), 6B, 23F (2), 9V (2), 19F] n (%)	Non-invasive isolate [n = 6B, 19F (5)] n (%)	P-value
Tetracycline	9 (100)	6 (100)	ND
Chloramphenicol	6 (66.7)	4 (66.7)	> 0.05
Penicillin	9 (100)	6 (100)	ND
Erythromycin	6 (66.7)	1 (16.7)	< 0.05
Trimethoprim/Sulfamethoxazole	5 (55.6)	1 (16.7)	< 0.05
Ceftriaxone	1 (11.1)	0 (0)	> 0.05
Cefotaxime	0 (0)	0(0)	> 0.05
Augmentin	5 (55.6)	0 (0)	ND
Ciprofloxacin	0(0	0(0)	ND
Laevofloxacin	0 (0)	0 (0)	ND

Table 7. Distribution of *S. pneumoniae* isolates by age among the infected patients.

Age group (year)	*S. pneumoniae,* n (%)^
1-2	2 (13.3)
3-5	5 (33.3)
6-13	4 (26.7)
14-19	1 (6.7)
20-40	1 (6.7)
41 and above	2 (13.3)
Total	15 (100)

^P<0.05 (1 – 5 vs. 6 – 13 or 41 and above age groups) according to Fischer exact test.

Table 8. Empirical antibiotic prescription rate among the patients studied

Antibiotic	Prescription^ n (%)
Penicillin	8 (19.5)
Trimethoprim/sulfametoxazole	11 (26.8)
Augmentin	6 (14.6)
*Ceftriaxone	4 (9.8)
Ciprofloxacin	5 (12.2)
[@]Chloramphenicol	5 (12.2)
[@]Gentamicin	3 (7.3)
Ampicillin	7 (17.1)
Ampicillin + Chloramphenicol	3 (7.3)
TMP/SMX + Chloramphenicol	2 (4.9)
TMP/SMX + Gentamicin	2 (4.9)
Ampicillin + Gentamicin	1 (2.4)

^Empirical prescription data were extracted for 41 patients. [@]Noted as ear drop applications. Ceftriaxone was administered empirically as an injection.

become increasingly important in South America as aetiologic agent of meningitis, bacteremia and sepsis (Camargos et al., 2006). Apart from the serotype 9V strains found in Lagos, the recovery of four other distinct serotypes (5, 6B, 19F and 23F), which has also been reported in previous studies, is a reflection of high level of serotype diversity among *S. pneumoniae* in circulation in Nigeria. Other serotypes that have been reported but not found in this study include serotypes 3, 4, 6A, 11, 14, 15C and 18C.

Our isolates were further differentiated into 6 RAPD types and 3 proteotypes, suggesting that they are clonally diverse. However, the clustering of serotypes 19F and 23F isolates within the same RAPD type and the presence of non-typeable serotype 9V isolates demonstrates the limitations of RAPD for better epidemiological characterization of *S. pneumoniae* isolates in this environment. Therefore, for better phylogenetic grouping of *S. pneumoniae* and improved understanding of serotype switching, typing techniques such as multilocus sequence typing (MLST) are required. The use of antibiotics remains an important component of therapeutic management of patients infected with *S. pneumoniae*. In this study, all our isolates were resistant to penicillin by disk diffusion assay but with serotype 9V and 1 of the 3 recovered serotype 5 isolates actually toelicit absolute resistance by MIC. Our finding agrees with the baseline fact that oxacillin disk is inadequate in distinguishing intermediate resistance from absolute resistance of *S. pneumoniae* to penicillin (Chwla et al., 2010). The observed 100% resistance rate, each for penicillin and tetracycline and 40% for trimethoprim/ sulfamethoxazole were higher than 36, 21 and 14% reported for these antibiotics by Fashae et al. (2002) during a *S. pneumoniae* outbreak in Ibadan in 2002, but were comparable with respect tetracycline resistance by invasive isolates reported by Falade et al. (2009) in 2009. These workers also reported 100% resistance rate to trimethoprim/sulfamethoxazole by these pathogens. On the contrary, all the invasive isolates recovered by Obaro et al. (2011) in North Central Nigeria in 2011 were sensitive to augmentin and ceftriaxone as reported previously by Fashae et al. (2002).

Meanwhile, in Jos, also within the north central Nigeria,

resistance rates of 34.2% to erythromycin, 29.7% to penicillin and 10.8% to ciprofloxacin were reported for nasopharyngeal isolates of *S. pneumoniae* by Kandakai-Olukemi and Dido (2009). Furthermore, Ndip et al. (1995) had previously reported absolute sensitivity to penicillin and erythromycin by *S. pneumoniae* isolates causing otitis media in Lagos in 1995. In a hospital-based study by Akanbi et al. (2004) in 2002 in Ilorin, Nigeria, the recovered *S. pneumoniae* isolates were resistant to all the tested antibiotics, including penicillin (83%), erythromycin (56.6%), ceftriaxone (28%), ciprofloxacin (20%) and ampicillin (73.8%). In this study, resistance rates of 66.7, 46.7, 33.3 and 6.7% to chloramphenicol, erythromycin, augmentin and ceftriaxone were elicited by our isolates with all being sensitive to ciprofloxacin, levofloxacin and cefotaxime. Apart from confirming that antibiotics vary in their efficacy against *S. pneumoniae* according to site of colonization or infection, geographical location within a country and serotype affiliation (the latter was not shown by previous studies), our findings have further demonstrated changing trend in antibiotic susceptibility among the circulating serotypes of *S. pneumoniae* and confirm the need for continuous monitoring of *S. pneumoniae* for antibiotic resistance coupled with restricted use of antibiotics in the country (Agwu et al., 2006; Adeleye et al., 2008; Falade et al., 2009).

The extensive use, misuse and abuse of antibiotics have been identified as persistent promoting factors of drug resistance in developing countries of world, where access to drugs are poorly controlled and the level of self-medication remains high (Arikpo et al., 2011). In southern Nigeria, emergence of chloramphenicol and ampicillin resistant pathogens, including *S. pneumoniae*, *Klebsiella pneumoniae, M. pneumoniae,* and *Moraxella catarrhalis* responsible for neonatal meningitis was first reported in 1994, preceding the period of high use of chloramphenicol and ampicillin as essential medicines for the management of infectious diseases in the country (Akpede et al., 1994). In fact, empirical prescription is a standard clinical practice in Nigeria with the use of and antibiotics such as chloramphenicol and TMP/SMX as first line drugs in children under the integrated management of childhood illness (IMCI) scheme (Obaro et al., 201). Penicillin, erythromycin and trimethoprim/ sulfamethoxazole are also three distinct classes of antibiotics that are widely used prophylactically to prevent pneumococcal diseases and other opportunistic infections (e.g. *Pneumocystis carinii* infection) in Nigerian patients with sickle cell anaemia and HIV/AIDS (Grange et al., 2003). Therefore, our present antibiogram result, regarding these antibiotics is not surprising. After all, Adeleye et al. (2008) reported 100% resistance rate to TMP/SMX by *S. pneumoniae* isolates from HIV/AIDS patients in Lagos. The presence of substantial drug pressure by TMP/SMX in Nigeria may also be connected to the high use of the drug sulfamethoxazole-pyrimethamine for intermittent preventative treatment (IPT) of malaria in pregnant women (Agomo et al., 2009) and by self-mediation at home in malaria management (Iwalokun et al., 2011). All over the world, resistance rate to penicillin by *S. pneumoniae* has increased progressively since the first report in 1967 in Australia (Hansman and Bullen, 1967). Apart from HIV sero-positivity correlating with penicillin and TMP/SMX resistance by *S. pneumoniae* (Adetleye et al., 2008; Jenkins et al., 2008), other factors that have been identified by previous workers include prior use of β-lactam antibiotics, 3 months of hospitalization and a revious history of pneumonia (Crewe-Brown et al., 1997).

The present retrospective study has limitations in that most of these factors were not extracted for risk factor analysis. However, our empirical antibiotic usage data revealed empirical prescriptions of TMP/SMX, penicillin, augmentin and ceftriaxone to which resistance was observed among our isolates. This finding also confirms the role of antibiotic usage in the emergence and spread of drug resistant pathogens in endemic populations. Nigeria is one of your populations in which antibiotic usage without prescription and empirical prescription of antibiotics are high (Obaro et al., 2011). Therefore our findings warn against empirical prescriptions of antibiotics, particularly drugs like penicillin, TMP/SMX, other β-lactams antibiotics, tetracyclines and chloramphenicol against *S. pneumoniae* infections in these settings. In a situation where empirical therapy is inevitable, the use of third generation cephalosporins and fluoroquinolones such as ciprofloxacin and laevofloxacin is recommended in these settings. The possibility of using combination of two classes of antibiotics empirically is also advocated. But evidence based research is recommended to justify this. With regards to penicillin use, previous studies in other environments still support the use of this antibiotic against intermediate resistance serotypes based on the fact that penicillin is a class of antibiotics whose activity is time-dependent and with efficacy dependent on the duration of effective MIC between dosing. Since penicillin allows daily multiple dosing and is affordable, pharmacokinetics and pharmacodynamic data beyond MIC are urgently needed for its continued usage in our settings. It is equally important to monitor *S. pneumoniae* for emergence of new serotypes and incidence of serotype switching in our setting to guide the deployment of effective control measures. In this study, we found serotype 9V isolates which were not reported as invasive and non-invasive pathogens by previous workers (Adeleye et al., 2008; Obaro et al., 2011; Falade et al., 2009; Adetifa et al., 2012; Antonio et al., 2008). This suggests serotype evolution. Clinically, serotype 9V *S. pneumoniae* isolates were also found to be the most drug resistant, confirming the importance of serotyping in *S. pneumoniae* epidemiology and suggesting changing epidemiology of invasive *S. pneumoniae* infection coupled with the need

for continuous monitoring of these serotypes and emergence of new ones in Nigeria. Generally, the serotypes of *S. pneumoniae* recovered in this study are among the common 20 serotypes that account for over 70% of pneumococcal diseases due to *S. pneumoniae* worldwide. However, based on serotype composition, we found theoretical coverage rate of 80 and 100%, respectively for PCV-7 and PCV-13, suggesting that Nigeria will benefit more from PCV-13 than PCV-7 if adopted for routine vaccination to control pneumonia in the country. Currently, the global coverage rate of PCV-7 is less than 70% as it does reduce the burden of infections caused by *S pneumoniae* serotypes such as serotype 9V found in this study and serotypes 1, 3 and 4 reported previously in the country. Although PCVs are vaccines for children aged ≤ 6 years, children above 6 years but with underlying medical condition who have not had PCV coverage (Scott et al., 2011), the ability of this category of pneumococcal vaccines to elicit herd immunity is an indirect benefit for controlling pneumococcal diseases in adults, particularly those of extreme ages, who are equally susceptible as children to *S. pneumoniae* infections (Kim et al., 2010). This benefit is a possibility in Nigeria as for other endemic countries who have integrated PCV into their national immunization programmes (Antonio et al., 2008; Kim et al., 2010). One evidence–based explanation for this explanation is the recovery of serotypes covered by PCV-13 in adults with bacteremia, also seen in this study and other pneumococcal diseases reported previously by other workers from Nigeria and other countries of the world.

Based on our findings and despite the small sample size of our investigation, we report circulation of multiple serotypes and multi-drug resistant *S. pneumoniae* strains in south-West Nigeria and a potential public health benefit of PCV-13 if adopted for routine use and integrated into the national immunization programme of the country. The use of better typing platform such as MLTS is also recommended for better understanding of clonal diversity, dissemination and pathogenicity of *S. pneumoniae* at regional, national and country levels.

ACKNOWLEDGEMENTS

The authors wish to thank ISPPD-8 organizers for their travel award support to present part of this study in Brazil. Special thanks also to the technologists for their technical assistance and co-operation and study participants from whom *S. pneumoniae* isolates were recovered at the health facilities used for the study

REFERENCES

Adeleye A, Uju L, Idika N, Sobande O (2008). Cotrimoxazole Resistance in *Streptococcus pneumoniae* Isolated from Sputum of HIV-positive Patients. West Indian Med J. 57:497-499.

Adetifa IMO, Antonio M, Okoromah CAN, Ebruke C, Inem V, Nsekpong D, Bojang A, Adegbola RA (2012). Pre-Vaccination Nasopharyngeal Pneumococcal Carriage in a Nigerian Population. Epidemiol. Popul. Biol. PLoS ONE, 7:e30548.

Agomo CO, Oyibo WA, Anorlu RI, Agomo PU (2009). Prevalence of malaria in pregnant women in Lagos, South-West Nigeria. Korean J. Parasitol. 47:179-183.

Agwu E, Ohihion AA, Agba MI, Okogun GR, Okodua N, Tat Feng YM, Nwobu GO (2006). Incidence of *S pneumoniae* infections among patients attending tuberculosis clinics in Ekpoma, Nigeria. Shiraz E-Med. J. 7:1–11.

Akanbi II, Taiwo AA, Babatunde SS, Onilke SK, B.A, Abdulraheem IS (2004). Antibiotic susceptibility pattern of *Streptococcus pneumoniae* in Ilorin, Nigeria. Afr. J. Clin. Exp. Microbiol. 5:173–176.

Akinloye OM, Rönkkö E, Savolainen-Kopra C, Ziegler T, Iwalokun BA, Deji-Agboola MA, Oluwadun A, Roivainen M, Adu FD, Hovi T (2011). Specific viruses detected in Nigerian children in association with acute respiratory disease. J. Trop. Med. 2011:690286.

Akinsete I, Akanmu AS, Okany CC (1998). Spectrum of clinical diseases in HIV infected adults at the Lagos University Teaching Hospital: a five year experience (1992-1996). Afr. J. Med. Sci. 28:147–151.

Akpede O, Abiodun PO, Sykes M, Salami CE (1994). Childhood bacterial meningitis beyond the neonatal period in southern Nigeria: changes in organisms/antibiotic susceptibility. East Afr. Med. J. 71:14-20.

Akuse RM (1996). Variation in the pattern of bacterial infection in patients with sickle cell disease requiring admission. J. Trop. Pediatr. 42:318-323.

Antonio M, Dada-Adegbola H, Biney E, Awine T, O'Callaghan J, Pfluger V, Enwere G, Okoko B, Oluwalana C, Vaughan A, Zaman SMA, Pluschke G, Greenwood BM, Cutts F, Adegbola RA (2008). Molecular epidemiology of pneumococci obtained from Gambian children aged 2–29 months with invasive pneumococcal disease during a trial of a 9-valent pneumococcal conjugate vaccine. BMC Infect. Dis. 8:81.

Appelbaum PC (1992). Antimicrobial resistance in *Streptococcus pneumoniae:*an overview. Clin. Infect. Dis. 15:77–83.

Arikpo EA, Eja ME, Enyi-Idoh KH, Akubuenyi F, Ngang U, Akam C Ekomabasi I (2011). Patterns of antibiotics drug use in southern Nigerian communities. World J. Appl. Sci. Technol. 3:86-92.

Camargos P, Fischer GB, Mocelin H, Dias C, Ruvinsky R (2006). Penicillin resistance and serotyping of *Streptococcus pneumoniae* in Latin America. Paediatr. Respir. Rev. 7:209-214.

Chawla K, Gurung B, Mukhopadhyay C, Bairy I (2010). Reporting emerging resistance of *Streptococcus pneumoniae* from India. J. Global Infect. Dis. 2:10-14.

Clinical and Laboratory Standards Institute (CLSI) (2006). Performancestandard for antimicrobial disk diffusion tests; Approved standards. 9th ed. CLSI document. M2-M9. Wayne Pa: Clinical and Laboratory Standards Institute, 2006.

Crewe-Brown HH, Karstaedt AS, Saunders GL, Khoosal M, Jones N, Wasa A, klugman KP (1997). *Streptococcus pneumoniae* blood culture isolates from patients with and without human immunodeficiency virus infection: alterations in penicillin susceptibilities and in serogroups and in serotypes. Clin. Infect. Dis. 25:1165–1172.

Duarte RS, Barros RR, Facklam RR, Teixeira LM (2005). Phenotypic and Genotypic Characteristics of *Streptococcus porcinus* Isolated from Human Sources. J. Clin. Microbiol. 43:4592–4601.

Falade AG, Lagunju IA, Bakare RA, Odekanmi AA, Adegbola RA (2009). Invasive pneumococcal disease in children aged, 5 years admitted to 3 urban hospitals in Ibadan, Nigeria. Clin. Infect. Dis. 48:S190–196.

Fashae KF, Ogunsola FT, Salawu OM, Dada AO, Popoola O (2002). A possible outbreak of *Streptococcus pneumoniae* invasive infection in children in Ibadan, Nigeria. Afr. J. Med. Med. Sci. 31:141-143.

Grange AO, Iroha EO, Akinsulie AO, Temiye EO, Ezeaka VC, Adetifa IMO (2003). Guidelines for the management of HIV/AIDs in infants and older children in Lagos University Teaching Hospital. Nig. Qt. J. Hosp. Med. 13:8-13.

Hansman D, Bullen MM (1967). A resistant pneumococcus [letter].

Lancet. 2:264–265.
Hunter PR, Gaston MA (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26:2465-2466.
Iwalokun BA, Oluwadun A, Iwalokun O, Olukosi YA, Agomo PU (2011). Reduction in Febrile Episodes and Dynamics of Pyrogenic Threshold in Nigerian Children with *Plasmodium falciparum* Malaria. J. Paediatr. Infect. Dis. 6:185–194.
Jenkins SG, Brown SD, Farrell DJ (2008). Trends in antibacterial resistance among *Streptococcus pneumoniae* isolated in the USA: Update from PROTEKT US Years 1-4. Ann. Clin. Microbiol. Antimicrob. 7:1.
Kandakai-Olukemi YT, Dido MS (2009). Antimicrobial resistant profile of *Streptococcus pneumoniae* isolated from the nasopharynx of secondary school students in Jos, Nigeria. Ann. Afr. Med. 8:10-13.
Kim SY, Lee G, Goldie SJ (2010). Economic evaluation of pneumococcal conjugate vaccination in the Gambia. BMC Infect Dis. 10:260.
Lagunju IA, Falade AG, Akinbami FO, Adegbola R, Bakare RA (2008). Childhood bacterial meningitis in Ibadan, Nigeria--antibiotic sensitivity pattern of pathogens, prognostic indices and outcome. Afr. J. Med. Med. Sci. 37:185-191.
Merquior VLC, Peralta JM, Facklam RR, Teixeira LM (1994). Analysis of electrophoretic whole-cell protein profiles as a tool for characterization of *Enterococcus* species. Curr. Microbiol. 28:149-153.
Nagai K, Shibasaki Y, Hasegawa K, Davies TA, Jacobs MR, Ubakata K, Appelbaum PC (2001). Evaluation of PCR primers to screen *Streptococcus pneumoniae* isolates and b-lactam resistance, and to test the common macrolide resistance determinants. JAC 48:915-18.
National Committee for Clinical Laboratory Standards (1990). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Second Edition: Approved Standard M7-A2. NCCLS, Villanova, PA.
Ndip RN, Obi MC, Obi CL, Nwawolo C, Igumbor EO, Obi AA (1995). Antibiogram of bacterial isolates from cases of otitis media and lower respiratory tract infections. Afr. J. Med. Med. Sci. 24:353-357.
Obaro S (2009). Seven-valent pneumococcal conjugate vaccines for developing countries. Expert Rev. Vaccin. 8:1051-1061.
Obaro S, Lawson L, Essen U, Ibrahim K, Brooks K, Otuneye A, Shetima D, Ahmed P, Ajose T, Olugbile M, Idiong D, Ogundeji D, Ochigbo C, Olanipekun G, Khalife W, Adegbola R (2011). Community acquired bacteremia in young children from central Nigeria: A pilot study. BMC. Infect. Dis. 11:137.
Onche O (2009). Nigeria: Pneumonia Infects Six Million, Kills 200,000 Children Annually. Daily Independent, 21 Jan. p. 25.
Onyemelukwe GC, Greenwood BM (1982). Pneumococcal serotypes, epidemiological factors and vaccine strategy in Nigerian patients. J. Infect. 5:157–163.
Salo P, Ortqvist A, Leinonen M (1995). Diagnosis of bacteremic pneumococcal pneumonia by amplification of pneumolysin gene fragment in serum. J. Infect. Dis. 171:479–482.
Scott P, Rutjes AW, Bermetz L, Robert N, Scott S, Lourenço T, Egger M, Low N (2011). Comparing pneumococcal conjugate vaccine schedules based on 3 and 2 primary doses: systematic review and meta-analysis. Vaccine 229:9711-9721.
Tracey J, Coffey TJ, Daniels M, Enright MC, Spratt BG (1999). Serotype 14 variants of the Spanish penicillin- resistant serotype 9V clone of *Streptococcus pneumoniae* arose by large recombinational replacements of the cpsA-pbpla region. Microbiology 145:2023-2031.
Uneke CJ, Ezeoha AE, Ndukwe CD, Oyibo PG, Onwe F (2010). Development of Health Policy and Systems Research in Nigeria: Lessons for Developing Countries' Evidence-Based Health Policy Making Process and Practice. Health Policy 6:e109–e126.
WHO-UNICEF (2010). Estimates of immunization coverage: the federal |republic of Nigeria. WHO pp. 1–13.
World Pneumonia Day Final Media Report (2010). Global coalition against childhood pneumonia. pp. 1–12.