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Prevalence and gene sequencing of extended spectrum β-lactamases producing *Salmonella enterica* serovar. Typhi from South-East Nigeria

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Enteric fever is a systemic disease classified into typhoid and paratyphoid fever. Fluoroquinolones and third generation cephalosporins are usually the drugs of choice in the management of *Salmonella* infections. Previous reports have indicated common occurrence of multi-drug resistance (MDR) including resistance to β-lactams and fluoroquinolones. However, there is paucity of information on the genetic determinants of resistance to β-lactam and fluoroquinolones from *S. enterica* in Southeast Nigeria. *Salmonella enterica* serovars were identified and screened (25 each from unrelated patients from the four hospitals). Resistant isolates were screened for ESBL phenotypically. Genomic and plasmid DNA were extracted by boiling and alkaline lysis, respectively. PCR amplification of bla_{TEM}, bla_{SHV} and bla_{CTX-M}, among the ESBL positive isolates and sequencing of the Quinolone Resistance Determining Regions (QRDR) on fluoroquinolone resistant isolates were determined. Of the 100 isolates, thirty six of the MDR isolates produced ESBL phenotypically, of which 13 were bla_{CTX-M} positive. DNA sequencing revealed single point mutations in *gyrA* at amino acid positions Asp-87-Gly, Asp-87-Asn and Ser-83-Tyr in 55 (68.8%), and double mutation in *parC* at positions Asp-87-Gly in 14 (17.5%). Mutations in *gyrA*, *parC* genes, and chromosomal bla_{CTX-M} were responsible for the fluoroquinolones and cephalosporins resistance, respectively in some of the *Salmonella enterica* from Southeast Nigeria. QRDR of the *gyrA* gene of the isolates sequenced showed reduced susceptibility to some fluoroquinolone. The taxonomic and neighbouring trees of similar species causing infection worldwide were identified, and hence, alleviates the fear of easy spreading of quinolone and cephalosporin resistant isolates.

Key words: Quinolone Resistance Determining Regions (QRDR), ESBL, *S.enterica,* mutation.

INTRODUCTION

Enteric fever is a systemic disease classified into typhoid and paratyphoid fever (Elumalai et al., 2014). Paratyphoid fever is a less severe type of enteric fever caused by *Salmonella* Paratyphi A, B and C (Cheesbrough, 2006 ; CLSI, 2011).

Typhoid fever being a systemic infection caused by *Salmonella enterica* serotype Typhi is a highly adapted human specific pathogen which possesses remarkable

mechanism for persistence in host as reported by Onyenwe et al. (2011). It estimates for approximately 5.4 million cases worldwide per year (Elumalai et al., 2014). Typhoid fever is severe and caused by Gram negative bacterium *S. enterica* serovar Typhi (Elumalai et al., 2014). It can be transmitted via contaminated food, water and is mainly present in area with poor hygiene or low socioeconomic status (Mweu and English, 2008). According to the World Health Organization (WHO), they estimated that typhoid fever accounts for 222,000 deaths and 21 million infections annually on a global scale (WHO, 2015). Africa is classified under region with medium incidence rate per year (10-100/100,000 cases/year) centered on a 22 community-based incidence study with only three African countries included in the analysis (Crump et al., 2004). As of 2013, the annual mortality rate was reported at 2.8 per 100,000 person in Africa with 2.5 per 100,000 person reported for Nigeria (Health Grove, 2018). These numbers make typhoid fever an infectious disease worth investigating for better treatment plan and management.

The standard treatment for typhoid fever is the use of antibiotics which target the *Salmonella* bacteria (DrugBank, 2009). In the 1990s, there was the introduction of chloramphenicol which posed as the first line of treatment for typhoid fever. However, the development of resistance has led to multiple use of antibiotics including Co-trimaxazole, Ampicillin and third generation Fluoroquinolones and Cephalosporins. These antibiotics have contributed to reducing the mortality rate from 20 to 1-2%, according to Wain et al. (2015). Nevertheless, multidrug resistant (MDR) strains emerged with extended-spectrum β-lactamases (ESBLs) production affecting the effective treatment of *Salmonella* Typhi (Wain et al., 2015).

ESBLs are enzymes belonging to the β-lactamase group. They cause resistance to most β-lactam antibiotics including Cephalosporins, Penicillin, Monobactams and even early generation Cephalosphorins. ESBLs are divided into three main groups comprising TEM, SHV and CTX-M (Pitout et al., 2008). Previous studies indicated that the SHV family is derived from the *Klebsiella* species (Pitout et al., 2008), and are capable of hydrolysing first 3 generation Cephalosporins and Penicillin (D'Angelo et al., 2016). TEM are derived from *Escherichia coli* and have resistance against Penicillin, Ampicillin and first-generation Cephalosporin to extended spectrum Cephalosporins (D'Angelo et al., 2016). The CTX-M group is identified as the most prevalent type of ESBLs from *Salmonella enterica* (Pitout et al., 2008) and as their name suggest, they are the most powerful hydrolytic activity against CTX (D'Angelo et al., 2016). Also another study have however shown that majority of ESBLs in *Salmonella* are derived from TEM and SHV β-lactamasefamily (Elumalai et al., 2014). *Klebsiella pneumoniae, E. coli,* and *Klebsiella oxytoca* are the most commonly studied ESBL-producing organism due to the ability of easily identifying the presence of β-lactamase (D'Angelo et al., 2016). Thus ESBLs confer resistance to these antibiotics and related oxyimino-beta lactams (Schneider, 2008). In typical circumstances, they are derived from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases. However, there is infrequency when it comes to investigating *in-vitro* antibiotic combination therapy in ESBL *S.* Typhi infection. Thus, this study is aimed at investigating the prevalence and gene sequencing of ESBLs producing *S. enterica* serovar. Typhi from South-East Nigeria.

MATERIALS AND METHODS

Test organism

A total of 150 isolates of *S. enterica* were randomly collected from the Routine Section of the Medical Microbiology Laboratory in four hospitals from Southeast part of Nigeria. The isolates were from patients diagnosed with enteric fever that visited the four hospitals. Thus, the codes were designated as O for isolates from Owerri, U for isolates from Umuahia, E for isolates from Enugu and A for isolates from Abakaliki, accordingly, between June and October, 2017.

After investigation, 100 *S. enterica* serovars were identified and screened (25 each from unrelated patients from the four hospitals) by standard biochemical characterization, using microbact® identification kit-12E (oxoid-England). The antibiotic- sensitivity screening of the isolates (using McFarland standard of 0.5 ml dilution) were carried out by the multidisc agar diffusion method and E-test (Oxoid, India) as described by Onyenwe et al. (2012) on 20 ml molten Muller Hilton agar using antibiotics disc (oxoid, India), with a standard ATCC 14028 strain of *S. enterica* serovar Typhi. Members of the family Enterobacteriaceae that commonly express plasmid-encoded β-lactamases are TEM-1, TEM-2, and SHV-1 (Philippon et al., 2002; George et al., 2005).

Antimicrobial sensitivity testing

The screening for antimicrobial activity was carried out by the single disc agar diffusion method as described in Onyenwe *et. al.,* (2011). The zones of growth inhibition were interpreted using CLSI breakpoint as standard zones depending on the antibiotics used (CLSI, 2011; CLSI, 2014).

Beta-lacatamase production test using Nitrocefin sticks

The nitrocefin™ sticks (oxoid, India) is a chromogenic

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Table 1. Biochemical authentication of *Salmonella enterica* Serovar. Typhi

Biochemical test [99.8 (%) based on the microbact software]	Probability rate of colour/reaction
Oxidase	Yellow -
Lysine decarxylase	Yellow +
Glucose fermentation	Yellowish +
HS_2	Black deposit +
Mannose fermentation	Yellowish +
Catalase	Effervescent bubbles +
Nitrate utilization or reduction (ONPG)	Red +
Indole	Red -
Urease	Orange -
Simone Citrate utilization	Blue-green +
Voges Proskauer	Pink -
Trypton Deaminase	Yellowish brown -
Gram reactions under microscope	Tiny short negative rods

+ = Positive reaction**, - =** Negative reaction.

cephalosporin β-lacatamase indicator in a container (Murray et al., 2006). The Nitrocefin sticks were allowed to reach room temperature after removing it from the freezer. Then a well separated representative colony from the primary isolation medium was selected. The procedure was as described by Onyenwe et al. (2012). A positive reaction was shown by the development of a pink/red color. No color change was observed with organisms that do not produce beta-lactamase. The unused Nitrocefine stick was used as control.

Double disc synergy test (DDST) to detect ESBL's production

The test inoculum (0.5 McFarland turbidity equivalent) was spread onto Mueller-Hinton agar (MHA, Oxoid, India) using a sterile cotton swab. A disc of 20 µg Amoxycillin + 10 µg Clavunalic acid was placed on the surface of MHA; then discs of Ceftriaxone (30 µg), Ceftazidime (30 µg) and Cefotaxime (30 µg) (Oxoid, India) were kept around it in such a way that each disc was at distance ranging between 10 and 15 mm from the amoxil-clavulanic acid disc (centre to centre). The plate was incubated at 37°C overnight. The organisms were considered to be producing ESBL when the zone of inhibition around any of the broad-spectrum cephalosporin discs showed a clear-cut increase towards the amoxil-clavulanic acid disc (Onyenwe et al., 2011; Yah, 2010).

PCR amplification and agarose gel electrophoresis

The method was as described by Onyenwe et al. (2012). The purified DNA template in PCR Eppendorf tubes (2.0 µl DNA) was mixed with the Master Mix ready to load (HOT FIREPol ® DNA polymerase with 2.5 mM $MgCl_2$, Solis Biodyne). The mixture of primers, DNA and the Master Mix were vortexed, to mix and then centrifuged before introducing it into the PCR machine (Eppendorf-Germany). The PCR products were then taken for electrophoresis, after amplification (CBS Scientific Company Inc.) on the agarose gel at 80 to 100 V and finally viewed on the UV light for visible amplified image of the genes. A DNA-marker of 100 base pairs was used.

The thermo cycling condition (Solis biodyne 5x FIREPol) Master Mix Ready to load used for ESBL $Bla_{ctx\text{-}M}$ was 30 to 35 cycles of 95°C for 30 s, 72°C for 1 min, 66.2 for 1 min, 72°C for 1 min, 95°C for 30 s (PCR timing 1.38-2.58 h). The $Bla_{ctx\text{-}M}$ universal primers used were $Bla_{ctx\text{-}M}$ - F(5'ATG TGC AGY ACC AGT AAR GTK ATG GC-3') and R(5'- TGG GTR AAR TAR GTS ACC AGA AYC AGC GG- 3') where R in the sequence is purine, Y is pyrimidine, and S is G or C (this design is to occupy for the ambiguity of sequence variation among the CTX-M types) (Moubareck et al., 2005).

RESULTS

A total of 100 isolates of *S. enterica* serovar. Typhi were obtained out of the 150 screened from the four different hospitals from the Southeast region of Nigeria between June and October, 2017. The isolates were identified by cultural and biochemical characteristics using Microbact identification kit® 2E, thereby identifying the isolate to be *S. enterica* serovar. Typhi. as shown in Table 1.

Table 2 shows that only 2 (8%) isolates out of the twenty-five isolates recovered in GOPD unit from Federal Medical Center Owerri harboured plasmids, while 20 (80%) isolates produced the conventional β-lactamase. It was also observed that isolates recovered from a female child of 2 years in the CHOP unit produced the conventional β-lactamase enzyme but was resistant to only 4 (28.6%) antibiotics used in this study, while an isolate recovered in the EPU unit from a female child of 1 year old was resistant to the 4 (28.6%) of the antibiotics used but produced no conventional β-lactamase and harboured no plasmid.

From the results obtained from the *S. enterica* isolates (Table 3) from Federal Medical Centre (FMC) Umuahia, only 3 (12%) harboured plasmid, while 18 (72%) of the isolates recovered produced the conventional β-lactamase enzyme. It was also observed that isolates recovered from 2 (8%) female children in the IPU produced β-lactamase, though no plasmids were found on them, they were resistant to only 5 (35.7) of the 14 antibiotic used.

Table 2. Beta-lactamase production and plasmid profiling of isolates of S*almonella enterica* from Federal Medical Center Owerri, in relation to gender distribution.

Org. code [S/N]	Unit/Dept.	Sex	Age (years)	B-Lactamase production	Plasmid/Mwt.	Antibiotics resistant (%)
O1	IPU	M	Adult	+	-	5 (35.7)
O2	GOPD	F	20	+	+	2 (14.3)
O3	GOPD	F	Adult	+	-	2 (14.3)
O4	GOPD	F	26	-	-	3 (21.4)
O5	GOPD	F	22	+	-	7 (50.0)
O6	GOPD	F	Adult	+	-	4 (28.6)
O7	GOPD	F	Adult	+	-	3 (21.4)
O8	GOPD	F	Adult	+	-	2 (14.3)
O9	GOPD	F	Adult	+	+	8 (57.1)
O10	GOPD	M	Adult	+	-	7 (50.0)
O11	NHIS	F	Adult	+	-	9 (64.3)
O12	GOPD	M	Adult	+	-	9 (64.3)
O13	NHIS	F	Adult	+	-	7 (50.0)
O14	GOPD	M	Adult	+	-	10 (71.4)
O15	GOPD	F	50	+	-	6 (42.9)
O16	GOPD	F	50	+	-	7 (50.0)
O17	NHIS	M	Adult	-	-	8 (57.1)
O18	GOPD	F	65	-	-	10 (71.4)
O19	NHIS	F	Adult	+	-	10 (71.4)
O20	IPU	M	Adult	+	-	8 (57.1)
O21	IPU	M	Adult	+	-	5 (35.7)
O22	IPU	F	Adult	-	-	3 (21.4)
O23	EPU	F	1	-	-	4 (28.6)
O24	NHIS	M	Adult	+	-	6 (42.9)
O25	CHOP	F	2.5	+	-	4 (28.6)

F: Female, M: Male, NHIS: National Health Insurance Scheme, IPU: In-Patient Unit, GOPD: General Out Patient Department, CHOP: Children Out Patient, EPU: Emergency Patient Unit. Adult (includes male and female between 18 and above, whose actual age was not determined), Nil=Negative.

Results from University of Nigeria Teaching Hospital (UNTH) Enugu (Table 4) showed that there were no plasmid recovered from the 25 isolates but 20 (80%) of the isolates produced the conventional β-lactamase enzyme. It was also observed from this study that an isolate recovered from a child of 10 years from CHOP unit produced no conventional β-lactamase, harboured no plasmid but was resistant to 9 (64.3%) of the antibiotics used for the study.

Results on the *S. enterica* recovered from Federal Medical Centre (FMC) Abakaliki (Table 5) showed that 4 (16%) of the isolates harboured plasmids, with one of the isolates habouring the plasmid coming from a female child of 6 years old in the NHIS unit. Analysis also showed that the isolates recovered from the female child of 6 years as described earlier produced the conventional β-lactamase and was resistant to 10 (71.4%) of the 14 antibiotics used in this study. Almost all the isolates recovered from this part of Southeast (Abakaliki) in this study, produced the conventional β-lactamase. This could be as a result of the fact that the isolates have been recovered from IPU and GOPD unit of the hospital. It was also observed that one isolate recovered from a female patient in the IPU unit harboured no plasmid, but produced the β-lactamase enzyme and was resistant to only one antibiotic used in this study (Table 5). The resistance by *S. enterica* on the number of antibiotics tested was found in FMC Abakaliki to be the highest in the region (South-East Nigeria).

From Table 6, analysis showed the hospital record of the age range of individuals (patients) from whom *S. enterica* isolates were collected in this study. A total of 9 (9%) of the patients as shown on the record sheet of the hospital had plasmid DNA harbored in the isolates recovered from them (both male and female). While 55 (55%) of the total isolates showed positive *GryA* (male and female), 14 (14%) of the isolates had *ParC* (male and female), and 13 (13%) positive Bla_{CTX-M} type gene (male and female), and non harboured *bla*-SHV and *bla*-TEM, in the genomic DNA of the isolates *S. enterica*

Table 3. Beta-lactamase production and plasmid profiling of isolates of *Salmonella enterica* from Federal Medical Center Umuahia, in relation to gender distribution.

Org. code [S/N]	Unit/Dept.	Sex	Age (years)	B-Lactamase production	Plasmid/Mwt.	Antibiotic resistant (%)
U26	IPU	F	Adult	-	-	5 (35.7)
U27	IPU	F	Adult	+	-	6 (42.9)
U28	IPU	M	Adult	+	+	7 (50.0)
U29	IPU	F	Adult	-	-	3 (21.4)
U30	IPU	F	Adult	-	-	6 (42.9)
U31	IPU	F	28	+	-	1 (71.0)
U32	IPU	F	Adult	+	-	6 (42.9)
U33	IPU	M	Adult	+	-	7 (50.0)
U34	IPU	F	Adult	-	-	4 (28.6)
U35	IPU	M	19	+	-	5 (35.7)
U36	IPU	M	Adult	+	-	4 (28.6)
U37	IPU	F	13	-	-	5 (35.7)
U38	IPU	F	Adult	-	-	6 (42.9)
U39	IPU	M	24	+		6 (42.9)
U40	IPU	F	Adult	+	+	11 (78.5)
U41	IPU	F	3	+	-	5 (35.7)
U42	IPU	F	Adult	-	-	4 (28.6)
U43	IPU	M	Adult	+	+	5 (35.7)
U44	IPU	F	Adult	+	-	9 (64.3)
U45	IPU	M	Adult	+	-	3 (21.4)
U46	IPU	F	4	+	-	5 (35.7)
U47	GOPD	F	Adult	+	-	4 (28.6)
U48	GOPD	M	44	+	-	7 (50.0)
U49	IPU	F	Adult	+	-	11 (78.5)
U50	IPU	M	Adult	+	-	7 (50.0)

F: Female, M: Male, NHIS: National Health Insurance Scheme, IPU: In-Patient Unit, GOPD: General Out Patient Department, CHOP: Children Out Patient, EPU: Emergency Patient Unit. Adult (includes male and female between 18 and above, whose actual age was not determined), Nil=Negative. Mwt., molecular weight.

recovered from various age groups as shown in Table 6. Analysis revealed that the highest number of *S. enterica* isolates were recovered from patients between 18 and above, followed by the ages between 21 and 30 and the least ages between 0 and 10 years.

Figure 1 shows the graphical representation of the genetic constituents of the isolates of *S. enterica* and their resistance factors as related to each of the 5 selected antibiotics screened. Out of the 29 isolates of *S. enterica* resistant to Ciprofloxacin, only 15 produced mutation in *GyrA*, 8 produced double mutation in *ParC*, 1 produced Bla_{CTX-M} and three isolates harbored plasmids. Also, out of 22 isolates resistant to levofloxacin 9 produced mutation in *GyrA* genes, while *ParC*, Bla_{CTX-M}, and plasmids were produced by only two (2) isolates each. Other resistance gene produced by each *S. enterica* isolates resistant to other antibiotics such as ceftriaxone (CRO), cefotaxime (CTX-M), and amoxiclavulanic acid (AMC) are as shown in Figure 1.

Table 7 shows the result of the sequence alignment of the gene resulting from the PCR amplifications of the DNA complete genome of the isolate from the various hospitals (SO3, SO14, SA96, SU33, and SA98) as shown in Table 7. From Table 7, isolate SO3 had 95 and 94% maximum identity with the strains found in the gene bank (*S. enterica* subsp. enterica serovar. Typhimurium str. UK-1 chromosome, complete genome and *S. enterica* subsp. enterica serovar. Typhi str. CT18, complete genome) with the accession number NC016863 and NC_003198 after 100% blast hits (query coverage) on the BLAST software, respectively. Also, isolate SA98 (Table 7) had 99 and 94% identity with the strain found in the genebank (*S.* Typhimurium strain 580 *GyrA* gene, partial cds and *S. enterica* subsp. enterica serovar. Typhimurium strain ATCC 307) with the accession number EF059893.1 and CP009102.1, after 97% blast hits (query coverage) on the BLAST software. Another gene (isolate SO14) sequenced was found to be 89% identical to a strain, *S. enterica* subsp. enterica serovar Typhi strain B/SF/13/03/195, complete genome. Others

Table 4. β-lactamase production and plasmid profiling of isolates of S*almonella enterica* from University of Nigeria Teaching Hospital (UNTH) Enugu, in relation to gender distribution.

Org. code [S/N]	Unit/Dept.	Sex	Age (years)	B-Lactamase production	Plasmid/Mwt.	Antibiotic resistant (%)
E51	IPU	F	60	+	-	7 (50.0)
E52	GOPD	F	56	+	-	9(64.3)
E53	GOPD	M	Adult	+	-	8 (57.1)
E54	IPU	M	Adult	+	-	5 (35.7)
E55	IPU	F	Adult	+	-	7 (50.0)
E56	SKIN	M	80	+	-	6 (42.9)
E57	GOPD	F	56	+	-	6 (42.9)
E58	IPU	M	21	+	-	5 (35.7)
E59	IPU	M	Adult	+	-	4 (28.6)
E60	IPU	F	Adult	-	-	7 (50.0)
E61	CHOP	M	10	-	-	9 (64.3)
E62	GOPD	M	Adult	-	-	3 (21.4)
E63	GOPD	M	Adult	+	-	6 (42.9)
E64	GOPD	M	Adult	+	-	8 (57.1)
E65	GOPD	F	Adult	-	-	8 (57.1)
E66	GOPD	F	40	-	-	13 (92.9)
E67	GOPD	F	60	+	-	11 (78.5)
E68	GOPD	F	101	+	-	10 (71.4)
E69	GOPD	M	21	+	-	12 (85.7)
E70	GOPD	F	27	+	-	7 (50.0)
E71	IPU	F	76	+	-	9 (64.3)
E72	IPU	M	45	+	-	11 (78.5)
E73	GOPD	M	33	+	-	8 (57.1)
E74	GOPD	F	Adult	+	-	6 (42.9)
E75	GOPD	M	60	+	-	13 (92.9)

F: Female, M: Male, NHIS: National Health Insurance Scheme, IPU: In-Patient Unit, GOPD: General Out Patient Department, CHOP: Children Out Patient, EPU: Emergency Patient Unit. Adult (includes male and female between 18 and above, whose actual age was not determined), Nil=Negative. Mwt., molecular weight.

are as shown in Table 7.

DISCUSSION

ESBLs are enzymes belonging to the β-lactamase group. They cause resistance to most β-lactam antibiotics including Cephalosporin, Penicillin, Monobactams and even early generation Cephalosporin and Ciprofloxacin. Analysis in this study revealed that the cause of resistant to these antibiotics was not only as a result of plasmids but also some plasmids mediating enzymes and chromosomally mediating enzymes. Enzymes such as β-lactamase enzymes and extended spectrum β-lactamase enzymes were detected to have initiated some forms of resistant (either enzyme inhibition or resistant mutants) to these antibiotics especially the Cephalosporines and Floroquinolones which has been recommended as a replacement by most authorities for the treatment of typhoid fever. Many reports have been put forward by several authors such as Brusch et al. (2010) that Floroquinolones are highly effective against susceptible organism, yielding a better cure rate than Cephalosporins, but that resistance to first generation Floroqninolones is widespread in many parts of Asia (Bruschet al., 2010). Furthermore, they stated that in recent years, third generation Cephalosporins have been used in regions with high Floroquinolones resistance rates, particularly in South Asia and Vietnam (Brusch et al., 2010) but much has not been reported in this part of Nigeria, especially in this study. Furthermore, in this study, analysis showed that both the third generation cephalosporin and the fluoroquinolones are still maintaining their choice as a replacement for the treatment of phenotypically detected chloramphenicol-resistant strain of *S. enterica* serovars of Typhi in this part of Nigeria.

Analysis from this study showed that 13 (13%) positive bla_{CTX-M} type gene were being harboured in the chromosomal DNA of the isolates *S. enterica* recovered

Table 5. β-lactamase production and plasmid profiling of isolates of S*almonella enterica* from Federal Medical Center (FMC) Abakiliki, in relation to gender distribution.

Org. code [S/N]	Unit/Dept.	Sex	Age (years)	B-Lactamase production	Plasmid/Mwt.	Antibiotics resistant (%)
A76	GOPD	M	Adult	+ve	+ve	12 (85.7)
A77	GOPD	M	20	+	-	14 (100)
A78	EPU	F	59	+	-	13 (92.7)
A79	IPU	M	24	+	-	10 (71.4)
A80	IPU	M	Adult	+	-	13 (92.7)
A81	NHIS	F	6	+	+	10 (71.4)
A82	GOPD	M	Adult	+	-	10 (71.4)
A83	GOPD	F	58	+	+	12 (85.7)
A84	IPU	F	68	+	-	13 (92.7)
A85	IPU	F	Adult	+	+	10 (71.4)
A86	GOPD	M	34	+	-	12 (85.7)
A87	GOPD	F	Adult	+	-	9 (64.3)
A88	IPU	M	Adult	+	-	11 (78.5)
A89	GOPD	F	29	+	-	6 (42.9)
A90	HNIS	M	39	+	-	9 (64.3)
A91	IPU	F	39	-	-	8 (57.1)
A92	IPU	F	20	+	-	1 (7.1)
A 93	IPU	M	34	-	-	9 (64.3)
A 94	GOPD	F	39	+	-	11 (78.5)
A 95	IPU	M	25	+	-	7 (50.0)
A 96	IPU	F	Adult	+	-	6 (42.9)
A 97	IPU	F	Adult	+	-	6 (42.9)
A 98	NHIS	F	Adult	+	-	8 (57.1)
A 99	IPU	F	Adult	+	-	9 (64.3)
A100	IPU	F	Adult	-	-	12 (85.7)

F: Female, M: Male, NHIS: National Health Insurance Scheme, IPU: In-Patient Unit, GOPD: General Out Patient Department, CHOP: Children Out Patient, EPU: Emergency Patient Unit. Adult (includes male and female between 18 and above, whose actual age was not determined), Nil=Negative. Mwt., molecular weight.

Table 6. Distribution of resistant determinants of S*almonella enterica* in relation to patient's age from the Southeast region of Nigeria.

S/N	Age limits	No. of patients	Presence of plasmids		Presence of Gyr A		Presence of Par C		Presence of Bla_{CTX-M}		*Bla*TEM/ BlaSHV/Q*nrB*
	(years)	%	(M)	(F)	(M)	(F)	(M)	(F)	(M)	(F)	
1	0-10	6	0	1	1	3	0	0	0	0	Not detected
2	11-20	5	0	1	2	2	1	0	0	0	Not detected
3	21-30	11	0	0	5	1	1	0	1	0	Not detected
4	31-40	6	0	0	1	2	1	0	0	0	Not detected
5	41-50	4	0	0	0	2	1	0	1	0	Not detected
6	51-60	6	0	1	0	1	1	0	0	0	Not detected
7	61-70	2	0	0	0	0	0	1	0	0	Not detected
8	71-Above	3	0	0	0	0	0	0	0	0	Not detected
9	Adult (18-Above)	57	3	3	15	19	1	7	6	5	Not detected
Total		100	3	6	24	31	6	8	7	6	Nil in all
Total percentages		-	9 (9%)		55 (55%)		14 (14%)		13 (13%)		-

M: Male Patients, F: Female Patients , Adult (18- Above): Patients that are up to 18 years and above and refuse to disclose their actual age, *GyrA*: Gyrase A enzyme, Bla_{CTX-M} : Beta lactamase cefotaxime-M class enzyme, *bla*-SHV: Beta-lactmase Sulfhydryl variable, *bla*-TEM: Beta-lactamase Temoniera, *ParC*: Topoisomerase iv enzyme. The *QnrB* and other ESBL checked were not detected on all the isolates (*bla*-SHV, *bla*-TEM).

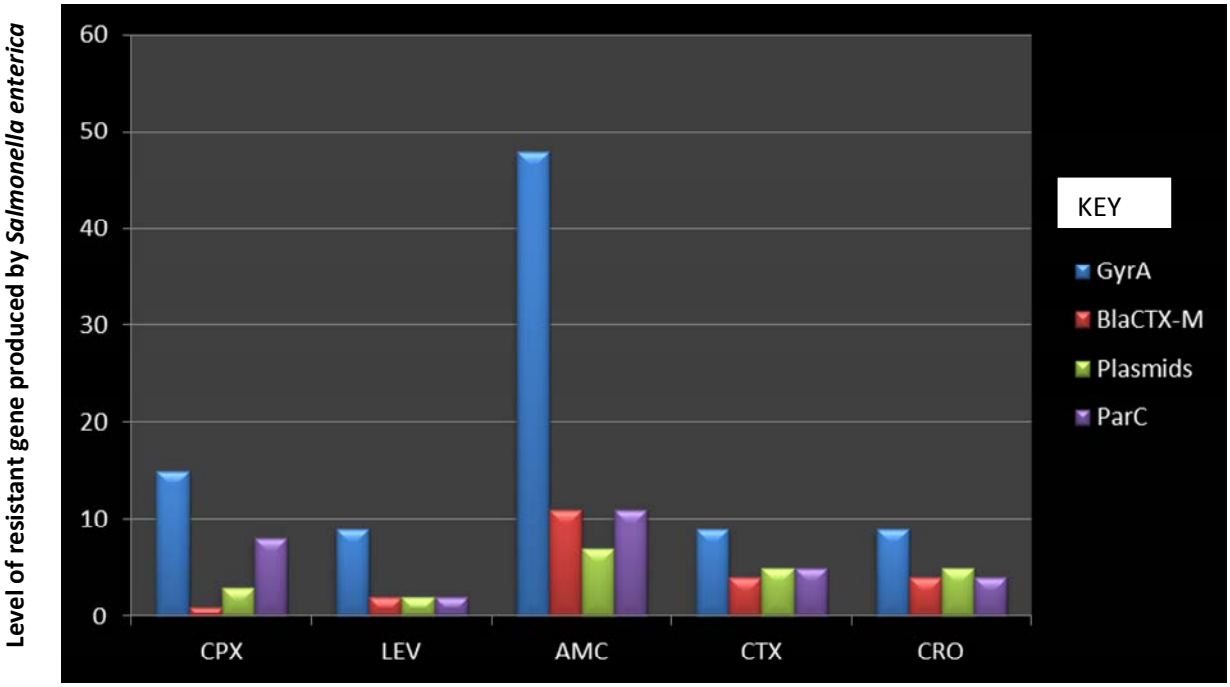

Figure 1. Graphical representation of the genetic constituents, the number of antibiotics and resistant pattern of isolates that produced plasmids or a type of mutation in a gene detected using PCR amplification, out of the total isolates resistant to each antibiotic.

from various age groups as shown in the Table 2. Though not all the isolates were β-lactamase positive, as only 80% of the isolates produced β-lactamases, while 36% of the isolates were positive to ESBLs. Also, it was discovered that only 28 isolates had both the conventional β-lactamases and the ESBL's (Tables 2 to 5). This trend of result could have been the reason why there was multiple resistances to these antibiotics used in this study. According to Yujuan and Ling (2006), Valverde et al. (2008) and Yah (2010) in their studies show resistance to broad spectrum β-lactams is highly mediated by ESBL enzymes, which has been increasing the world health problems in clinical settings.

According to Yah (2010), the plasmid-borne β-lactamases are also competent enough to hydrolyze β-lactam antibiotics. Also, according to Soge et al. (2005), the first CTX-M-type *β*-lactamases in Nigeria were identified as plasmid-encoded enzymes in clinical isolates from the Enterobacteriaceae, and all 30 isolates of *Klebsiella pneumonia* in their study produced at least one β-lactamase and 17 (57%) produced a CTX-M *β*-lactamase (Soge et al., 2005). Reports have also shown that the resistance of gastroenteric *Salmonella* strains to these antimicrobial agents is in large part due to the production of ESBLs encoded on plasmids as well as on the chromosome, as reported by David and Frank (2000), Yujuan and Ling (2006), Yah (2010) and Onyenwe et al. (2012).

According to the CLSI (2011) standard used for the sensitivity screening in this study, few of the isolates of *S. enterica* were sensitive to Chloramphenicol, though no molecular analysis was carried out to confirm the gene or enzyme responsible for it.

It is worthy to note that due to resistance and safety concerns, chloramphenicol should no longer be a first line agent for indication of *S. enterica* treatment in developing nation like Nigeria as seen in this epidemiological survey using phenotypic characterization. According to Hakanen et al. (2001), the emergence of antimicrobial resistance in any part of the world may have a global bearing and thus deserves universal attention.

In this study, at least 3 *S. enterica* serovars harboring plasmids were found to be resistant to some groups of antibiotics (SA81, SA83, and SA85 isolates) including 2 members of the Fluoroquinolone (Ciprofloxacin and Levofloxacin) without harboring any resistance gene or mutation in their *gyrA* and *parC* regions in the chromosomes, hence may be an indication of plasmid-mediated quinolone resistance (PMQR), no characterization was done on the gene. According to Hopkins et al. (2005) and Haugum et al. (2006), such resistance was reported only in *Klebsiella* and *E. coli*. Earlier studies have observed that in Salmonellae, the relative frequency of different mutations in *gyrA* was dependent on the quinolone antibiotics used for selection (Levy and Manshall, 2004). According to Lindtedt et al. (2004) it was discovered that a geographically dependent distribution of *GyrA* mutation was at codons 83 and 87 in

Table 7. BLAST analysis of the gene sequencing and alignment of S*almonella enterica* from South-East Nigeria.

Sequenced gene	Description of significant alignment	Accession No. of aligned gene	Max. score	Total score	Query coverage (%)	E-value	Max. Identity (%)
SO3	1.*Salmonella enterica* subsp. *enterica* serovar Typhimurium str. UK-1 chromosome, complete genome	NC016863.1	174	174	100	3e-43	95
	2.*Salmonella enterica* subsp. *enterica* serovar Typhi str. CT18, complete chromosome	NC_003198.1	169	169	100	2e-41	94
	3.*Salmonella enterica* subsp. *enterica* serovar Typhi str. Ty2 chromosome, complete genome	NC_004631.1	169	169	100	2e-41	94
SO14	1. *Salmonella enterica* subsp. enterica serovar Typhi strain B/SF/13/03/195, complete genome	CP012151.1	147	147	100	2e-35	89
	2.*Salmonella enterica* subsp. enterica serovar Typhi strain PM016/13, complete genome	CP012091.1	147	147	100	2e-35	89
SA96	1. *Salmonella enterica* subsp. enterica serovar Typhi isolate 9618-2K DNA gyrase subunit A (gyrA) gene, partial cds	AY302588.1	274	274	100	1e-73	92
	2.*Salmonella enterica* subsp. enterica serovar Typhi strain CMCSTDY DNA gyrase II (gyrA) gene, partial cds	KTI62085.1	268	268	100	6e-72	91
SA98	1. *Salmonella* Typhimurium strain 580 GyrA gene, partial cds	EF059893.1	363	263	97	1e-99	99
	2. *Salmonella enterica* subsp. enterica serovar Typhimurium genome assembly NCTC13348, chromosome	LN829401.1	307	307	97	5e-83	94
	3. *Salmonella enterica* subsp. enterica serovar Typhimurium strain ATCC 13311, complete genome	CP009102.1	307	307	97	5e-83	94
SU33	1. *Salmonella enterica* subsp. enterica serovar Typhi strain 21g DNA gyrase subunit A (gyrA) gene, partial cds	KC773840.1	246	246	96	2e- 65	92
	2. *Salmonella enterica* subsp. enterica serovar Typhi strain ST33 GyrA (gyrA) gene, partial cds	HQ176354.1	246	246	100	2e-65	91

SO3,SO14: Isolates from owerri, SA96: Isolates from Abakiliki, BLAST: Basic Local Alignment Search Tool, Query length of SO3: 109nts, Query I.D of SO3: /c/2251, Query length of SO14: 118nts, Query I.D of SO14: /c/_47953, Query length of SA96: 196nts, Query I.D of S96: /c/_82899; SA98: Isolates from Abakiliki, SU33: Isolates from Umuahia, BLAST: Basic Local Alignment Search Tool, Query length of SA98: 208nts, Query I.D of SA98: /c/_221473, Query length of SU33: 184nts, Query I.D of SU33: /c/_65033.

Salmonella hadar while Haugum et al. (2006) stated that the position and type of amino acid substitution in *gyrA* varied with the serovars. It has also been reported that among *S.* Typhi isolates obtained in the United States between 1999 and 2006, 43% were resistant to at least one antibiotic. In this study, 98% of the *S. enterica* serovars isolates were resistant to at least one of the 14 antibiotics tested. According to Hirose et al. (2002), Fluoroquinolones have become the first-line drugs for the treatment of typhoid, active against isolates of *Salmonella* species. However, several reports have declared treatment failures when these antimicrobials were used to treat *Salmonella* infections caused by strains with reduced Fluoroquinolone susceptibility (Hakanen et al., 2001), which is in line with the present study. Several clinical treatment failures after the administration of Ciprofloxacin and other Fluoroquinolone to patients with typhoid fever due to strains with decreased susceptibility to the Fluoroquinolone have also been reported by Threlfall and Ward (2001).

From this study, the result of the sequence alignment of the gene resulting from the PCR amplifications of the DNA complete genome of the isolate from the various hospitals (SO3, SO14, SA96, SU33, and SA98) as shown in Table 7, respectively. From Table 6, isolate SO3 had 95 and 94% maximum identity with the strains found in the gene bank (*S. enterica* subsp. enterica serovar. Typhimurium str. UK-1 chromosome, complete genome and *S. enterica* subsp. enterica serovar. Typhi str. CT18, complete genome) with the accession number NC016863 and NC_003198 after 100% blast hits (query coverage) on the BLAST software, respectively. Also, isolate SA98 (Table 7) had 99 and 94% identity with the strain found in the genebank (*S.* Typhimurium strain 580 *GyrA* gene, partial cds and *S. enterica* subsp. enterica serovar. Typhimurium strain ATCC 307) with the accession number EF059893.1 and CP009102.1, after 97% blast hits (query coverage) on the BLAST software. Another gene (isolate SO14) sequenced

was found to be 89% identical to a strain, *S. enterica* subsp. enterica serovar Typhi strain B/SF/13/03/195, complete genome. Others are as shown in Table 7. In this study, the Fast Minimum Evolution and Neighboring Joining Taxonomic tree of some strains sequenced including their relatedness was also found for isolates SO3, SO14, SU33, SA96, and SA98 (data not shown).

In this study, the mutations responsible for the Fluoroquinolone resistance in the *gyrA* and *ParC* genes of the *S. enterica* serovars were investigated. The sequences for the Quinolone Resistance Determining Region (QRDR) of the *gyrA* gene of the isolates which showed reduced susceptibility to some Fluoroquinolone were detected. There was single mutation at the Ser-83-Tyr, Ser-87-Gly and Ser-83-Phe, while some were found in Asp-87-Gly or Asp-86-Gly in *ParC* gene. The sequence analysis also revealed that some of the positions of the amino acids in the *gyrA* mutation were identified as Asp-87-Asn or at Ser-83-Tyr. The gene (from isolate SA98) sequence alignment revealed that the *S. enterica* characterized in this study had 99% similarity or identity to the typed gene of *S.* Typhimurium strain 580 *GyrA* gene, partial cds (conservative domains) and 94% identical to the American Typed Culture Centre (ATCC) strain known as *S. enterica* subsp. enterica serovar Typhimurium strain ATCC 307 found in the genebank, respectively. Also in this study, some NCTC13348 (National Centre for Typed Culture) strains chromosome were found to be 94% identical to the quinolone resistant gene of the isolate sequenced in this study. Another gene (isolate SO3 having point and double mutation at position 83 and 87 codon) sequenced was also observed to be 94% identical to a strain, *S. enterica* subsp. enterica serovar Typhimurium str. DT104, which is also similar to the gene of the clinical isolate reported by Hirose et al. (2002), which caused nosocomial infections in the United States with high level of Fluoroquinolone resistance as shown in this study, according to the Fast Minimum Evolution and Neighboring joining tree, taxonomic analysis (Zheng et al., 2000; Aleksandr et al., 2008).

Conclusion

Based on this study, it could be deduced that Chloramphenicol may no longer be a first line agent for any indication in both developed and developing nations like Nigeria. But, in low income countries like Nigeria, unfortunately, Chloramphenicol is still widely used mainly because it is cheap and readily available. The most serious adverse effect associated with its treatment is bone marrow toxicity. This may occur in two distinct forms bone marrow suppression, which is a direct toxic effect of the drug, usually reversible. Secondly, aplastic anaemia, which is idiosyncratic in nature (rare, unpredictable and unrelated to dose) are generally fatal according to Onyenwe et al. (2011). Arising from this study, Fluoroquinolones such as Levofloxacin should only be used in patients who have failed at least one prior therapy, and also reserved for use in seriously ill patients who may require immediate hospitalization as specified by Johnson and Johnson (2004, 2009) and Soge et al. (2005).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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