



## Plasmid Carriage and ESBL Production among *Salmonella* Enterica Serovar Typhi from Some Parts of Adamawa State, Nigeria

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### Abstract

Effective treatment of typhoid fever caused by *Salmonella enterica* serovar Typhi has been hampered by the emergence of multidrug resistant and extended spectrum beta lactamase producing strains thus making the organism an important public health pathogen especially in developing countries. This study was aimed at screening *Salmonella* ser. Typhi isolates from suspected enteric fever patients for the presence of plasmids as well as ESBL production. Eighty-four (84) *Salmonella* ser. Typhi isolates were obtained from blood and stool culture giving a prevalence of 17.5%. Results of susceptibility screening revealed that 37.5% of the isolates demonstrated ability to produce extended spectrum beta lactamase in vitro out of which 37.5% were isolates from Yola while 40% were isolates recovered from Gombi. Furthermore, 41(48.8 %) of the isolates from this study bore plasmids out of which 25(47.1 %) were from Yola metropolis while 13 (41.9 %) were from Gombi Local Government Area. The most common antibiotic resistant marker borne on the plasmids carried by *S. ser. Typhi* in the study area was resistance to AmpC Co (20) followed by resistance gene for ceftriaxone 10. Screening of the isolates for extended spectrum beta lactamase activity using the double disk synergy test revealed that 9 isolates namely; *Salmonella* ser. Typhi isolates GMB1, 6, 13, 26 and *Salmonella* ser. Typhi YLA 4, 11, 22, 31 and 42 showed positive ESBL activity. The PCR analysis revealed the presence of *Bla<sub>ctx</sub>* gene cluster in 4/25 (16%) of *S. Typhi* isolates. The detection of some strains with resistance to ceftriaxone as well as ability to produce the ESBL is an early warning sign indicating the need for more controlled use of this drug in the country especially in the study area.

**Keywords:** Plasmid, Resistance, ESBL

### INTRODUCTION

Typhoid fever is a systemic infection caused by *Salmonella enterica* serovar Typhi: a gram-negative peritrichously flagellated bacterium belonging to the family *Enterobacteriaceae*. Annually, *S. ser. Typhi* disease burden is put at 21.7 million illness and 217 000 fatalities globally (Marks *et al.*, 2010). Typhoid fever prevalence rates have been reported to be 42% in Owerri (Opera *et al.*, 2011), 80.1% in Abeokuta (Okonkwo *et al.*, 2010), 81.5% in Minna (Festus, 2014) and 18% in Adamawa Southern Senatorial District (Sale *et al* 2017). Before the advent of chemotherapy, the case fatality rate for typhoid fever was as high as 30% but with the introduction of chloramphenicol, ampicillin, or cotrimoxazole the mortality rate reduced to less than 1%. Sadly, by 1972, the emergence chloramphenicol-resistant *Salmonella* ser. Typhi were reported and by 1989, *S. Typhi* strains resistant to all the three first line drugs. This resistance to quinolone antibiotics is linked to the presence of non-transferable

have been reported in India, Pakistan, China, etc. This sad development meant that there were increased treatment failures and a rise in mortality and prolonged morbidity. Ballesté-Delpierre *et al.* (2016) attributed the resistance to be plasmid mediated thereby leading to the emergence of multidrug resistant strains. About 50-80% of *Salmonella* ser. Typhi isolates from China and the Indian subcontinents are now reported as multidrug resistant (MDR) and today such strains have been reported in many parts of the world including Nigeria. This development therefore poses a serious problem to chemotherapy because of narrow treatment options (Ballesté-Delpierre *et al.* 2016).

Following the emergence of MDR strains, fluoroquinolones such as ciprofloxacin and ofloxacin became the mainstay of typhoid fever chemotherapy but sadly also, there have been the emergence of *S. Typhi* isolates that are resistant to nalidixic acid and exhibiting reduced susceptibility to fluoroquinolones. chromosomal point mutation in the quinolone resistant determining region (QRDR) of the

bacterium although the resistance is sometimes reported to be plasmid mediated.

The emergence of Nalidixic acid resistant *Salmonella* ser. Typhi, (NARST) led to the use of third generation cephalosporin like ceftriaxone or cefotaxime treatment options of fluoroquinolones resistant typhoidal *Salmonella*. Unfortunately, there have been sporadic reports of high level resistance to ceftriaxone in *S. Typhi* due to the production of extended spectrum beta lactamase by these isolates (Mushtaq, 2006; Yoon *et al.*, 2009). This emergence of extended-spectrum beta-lactamase (ESBL) producing *S. ser. Typhi* adds to the challenge of typhoid fever chemotherapy because these isolates are resistant to most of the extended-spectrum cephalosporin and ceftriaxone in particular (Malini, 2009). This ESBL producing ability is reported to be encoded on a large, 80-kb to 300-kb plasmids that can be exchanged between bacterial species although a 23kb self-transmissible plasmid has been implicated in ESBL production among *Salmonella* ser. Typhi isolates (Akinyemi *et al.*, 2015).

Reviewed literature indicated that data on the plasmid carriage and ESBL production among *Salmonella* isolates have not been reported in the study area (Yola and Gombi local government areas of Adamawa State), thus the study was undertaken to provide a baseline information and reference for further studies. The aim of the study was to determine the plasmid carriage among *S. ser. Typhi* isolates as well as their extended spectrum beta lactamase producing abilities.

## MATERIALS AND METHODS

### Study Area

The study was conducted in Yola metropolis and Gombi local government area of Adamawa State. Yola was coined from the Fulfulde word Yoola, meaning 'Great Plain' and is the largest city, capital city and administrative centre of Adamawa State, Nigeria. It is located on Benue River and has an estimated population of 336,648. The geographical coordinates of Yola is 9°13'48"N and 12°27'36"E. Gombi on the other hand lies between coordinates 10.16 N and 12.74 E with an average population of 147, 787 Gombi. The occupation of the people in the study area is mainly agriculture (NPC, 2006). Low socioeconomic status, unhygienic practices, overcrowding, lack of proper sanitation and hygiene are common in the study

area. The MICS (2011) report showed that only 27.2% of the population in the study area have access to improved drinking water sources and sanitation and 33.9% of the population has been reported to practice open defecation, and another 25.2 % of the population reportedly use unimproved toilet facilities.

### Study Population

The subjects enrolled in this study included those that report to health facility in the study area with complains suspected to be enteric fever. They were also required to give their informed consent before they were enrolled.

### Study Sample

The MaCorr sample size calculator was used to compute the minimum sample size for the study at P=0.05 with the 2006 census population of Yola and Gombi as basis for sample size determination. The minimum number of samples required for the study was computed as 479

### Sample Collection

Blood (148 from Yola and 143 from Gombi) and stool (108 from Yola and 80 from Gombi) samples were collected from suspected enteric fever patients attending public and private hospitals and laboratories in Yola metropolis and Gombi and environ following standard laboratory procedure reported previously by Sale *et al.* (2017).

### Isolation and Identification of Isolates

Blood and stool samples were first enriched in selenite F broth for 24 hours at 37°C after which a loopful was inoculated on bismuth sulphite agar and incubated at 37°C for 24 hours. Colonies suspected to be *Salmonella enterica* serovar Typhi were subjected to the following biochemical tests; growth characteristics on kligler iron Agar, citrate utilization test, motility, indole, methyl red, voges Proskauer, citrate test using standard procedure describe by Chessbrough, (2006) and WHO (2003). Presumptive *S. ser. Typhi* isolates were confirmed by typing using standard antisera for *S. ser. Typhi*.

### Antibiotic Susceptibility Testing

The confirmed *Salmonella* ser. Typhi isolates were subjected to antimicrobial susceptibility testing using the disk diffusion method described by CLSI (2012). The antibiotic disk used for the test contained the following antibiotics chloramphenicol 30 µg, ampicillin, 10µg, amoxicillin 10µg, tetracycline 30µg, cotrimoxazole, 25 µg, nalidixic acid, 30µg, ofloxacin 5µg and ciprofloxacin 5µg.

### Plasmid Curing

Isolates showing resistance to some antibiotics in the antibiotic susceptibility test were screened for possible plasmid carriage using the plasmid curing protocol described by Mirmomeni *et al.* (2007). Briefly, 0.2 ml of overnight culture of *S. ser. Typhi* was inoculated into 5 ml nutrient broth containing 10% Sodium dodecyl sulphate (SDS), and incubated at 37°C for 24 hrs. After the incubation period, the broth culture was agitated to homogenize and then sub cultured onto Mueller Hinton agar plates. The resultant growth was screened for antibiotic resistance by the disk diffusion method as described earlier. Cured markers were determined by comparison between the pre and post curing antibiogram of isolates.

### Phenotypic Confirmatory Test for Esbl Producers

*Salmonella enterica* Typhi isolates showing resistance to third generation cephalosporin were further screened for extended spectrum beta lactamase production following the protocol reported by Babu *et al.* (2010). Briefly, *Salmonella enteric* serovar Typhi suspension in 0.85 % sodium chloride adjusted to 0.5 McFarland was inoculated onto Muller Hinton agar using a sterile Pasteur pipette. An antibiotic disc each of cefpodoxime (10 µg) and amoxicillin plus clavulanic acid (Augmentin, 30 µg) were placed on the surface of the inoculated medium with sterile forceps. The antibiotics were placed 20 mm apart from each other. The plate was then incubated at 37 °C for 24 hrs. An enhanced zone of inhibition of the cefpodoxime disc towards the Augmentin (30 µg) disc was considered as positive and noted as confirmed ESBL-producing organisms.

### DNA Extraction

Genomic DNA was extracted following the Qiagen DNA extraction protocol (Qiagen DNeasy, 2006). The spectrophotometer lens (nanodrop ND1000) was used to measure the amount and purity level of the DNA. A DNA sample with an optical density (OD) of 1 at 260nm corresponded to a DNA concentration of 50µg/ml of double-stranded DNA. The purified chromosomal DNA pellets were then transferred into a new tube and stored on ice. Purity levels were between 1.5-1.8 of 2 µl.

### PCR Analyses of Extended Spectrum Beta Lactamase Gene *Bla-ctx*

PCR Amplification of *Blactx* gene was performed using forward primer 5'-AAA AAT CAC TGC GCC AGT TC-3' and reverse 5'-AGC TTA TTC ATC GCC ACG TT-3 earlier reported by Smith *et al.* (2002) in a final volume of 25µl. The reaction mixture contained 5.5 µl

molecular grade water, 12.5 µl PCRmaster mix 2X (0.05 u/µl Taq DNA polymerase, reaction buffer, 4 mM MgCl<sub>2</sub>, 0.4 mM of each dNTP), 2.5µl of each primer and 2 µl of template bacterial DNA. Amplification reaction was carried on a GeneAmp System 2700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation (94 °C, 30 seconds), annealing (55 °C, 30 seconds) and extension (72 °C, 2 min 30 seconds) and a final extension for 10 minutes at 72 °C. The amplified products were separated by gel electrophoresis on 1.5% agarose at 100 volts and stained with ethidium bromide and visualised using Enduro gel documentation system. Molecular weight markers (50 base pairs) were used to determine the sizes of amplicons.

### RESULTS

Table 1 showed the distribution of *S. serovar Typhi* isolates from the study area by sample, age and gender. The culture results of the 479 stool and blood samples revealed that the overall occurrence of *Salmonella ser. Typhi* from the study area was 17.5% (Table 1). The results revealed that 20.7% of the samples from Yola and 13.9% of the samples from Gombi yielded growth of *Salmonella serovar Typhi* (Table 1). Furthermore, the finding showed that 40.1% of the *Salmonella ser. Typhi* isolated from the samples were from subjects belonging to age group 21- 30 years while the least occurrence was from subjects above 40 years of age with occurrence of 5.1%.

The male to female *Salmonella ser. Typhi* isolation ratio in the study area was 1.1:1 while the male to female ratio in Yola and Gombi was 1.52:1 and 1.07:1 respectively. Although overall more of the *Salmonella ser. Typhi* isolates were isolated from males than females and from Yola than Gombi the difference was not statistically significant at p=0.05 (Table 2). The recovery of the isolates in relation to sample collected revealed that more of the isolates were recovered from stool samples (above 30%) compared to blood samples with less than 10% isolation rate

Results of antibiotics susceptibility test of isolates revealed that the highest susceptibility index of 6.45 was observed for ofloxacin while the least susceptibility index was for ampicillin 0.11 (Table 3). High level of resistance was demonstrated *in vitro* by isolates from Gombi to ampicillin (94%), cotrimoxazole 25 (81%) and tetracycline 20 (65%) compared to isolates from Yola that showed high level resistance to ampicillin 47 (89%) and tetracycline 39 (74%)

(Table 3). A total of 23 isolates (27%) demonstrated in vitro resistance to ceftriaxone (Table 3). Greater susceptibility to ofloxacin 60 (71%) and Amoxicillin 55 (65%) was observed (Table 3).

Furthermore, results of antibiotics susceptibility test of isolates revealed that over 70% of isolates from Yola and Gombi were multi drug resistant with 49% and 55% being quinolone resistant respectively (Table 3). Resistance to third generation cephalosporin was highest in Gombi (32%) when compared to 25% in Yola (Table 3). In all there were 57 *Salmonella* ser Typhi resistance phenotypes out of the 84 isolates from the study area. The most common R type in the area was Amp, C, Co and Tet (5).

Results on investigation of the R- plasmid profile revealed that 51.2% of isolates from the study area possessed R Plasmids (Table 4). The distribution of the *S. ser. Typhi* isolates bearing R plasmid showed that 25 (47%) of isolates from Yola and 18 (58%) isolates from Gombi bore R plasmids. The most cured marker from the study was AmpCCo (20) which happens to be more in Gombi (11) than Yola. Plasmids bearing resistance to AmpCTet and AmpC Co-Ctx were only seen among isolates from Yola while isolates from Gombi harboured plasmids coding

for AmpTetNaCo and AmoCoTet resistance which was not observed amongst isolates from Yola (Table 4).

Screening of the isolates for extended spectrum beta lactamase activity using the double disk synergy test revealed that 9 isolates namely; *Salmonella* ser. Typhi isolates GMB1, 6, 13, 26 and *Salmonella* ser. Typhi YLA 4, 11, 22, 31 and 42 showed positive ESBL activity. Result from the plasmid curing experiment revealed that some of the resistance to third generation cephalosporin was plasmid borne as resistance to ceftriaxone was among the cured markers although some were chromosomal (Table 4).

Molecular screening of isolates for *Bla<sub>ctx</sub>* gene revealed that 4 out of 25 isolates (16%) screened for the gene were positive (lanes 12, 13, 21 and 24) (Figure 2). The isolates that bore the genes were; *Salmonella* ser. Typhi YLA 46 (lane 13) YLA41 and YLA 7 (lane 21 and 24). (Figure 2). One isolate from Gombi tested positive for chromosomal gene coding for *Bla<sub>ctx</sub>* that is *Salmonella* ser. Typhi GMB 17. The Resistance phenotypes of the isolates are Amx Amp Co Tet OfI Ctx Cp Na for *Salmonella* ser. Typhi YLA 46, Amp C Co Tet Ctx and Amp Tet OfI Ctx Cp Na for *Salmonella* ser. Typhi YLA 41, and YLA 7.

**Table 1: Distribution of *Salmonella* ser. Typhi based on age isolated from the different categories of sample types from the study area**

	Yola (256)		Gombi (223)		Total	P value
	Blood (148)	Stool (108)	Blood (143)	Stool (80)		
≤10 (n-32)	0	2	0	0	2(6.3)	
11-20 (n-93)	3	11	2	6	22(23.7)	
21-30 (n-132)	11	14	3	15	53(40.1)	
31-40 (n-105)	0	9	0	2	11(10.5)	
>40 (n-117)	1	2	0	3	6(5.1)	
<b>Total (%)</b>	<b>15(10)</b>	<b>38(35.2)</b>	<b>5(3.5)</b>	<b>26(32.5)</b>	<b>84(17.5)</b>	

**Table 2: Distribution of *Salmonella* ser. Typhi Isolates based on gender from study area**

	Yola		Gombi		Total	P value
	Male	Female	Male	Female		
S. Ser Typhi Present	32 (24.6)	21 (6.7)	16 (15.4)	15 (12.6)	84	0.7050
S Ser. Typhi Absent	98 (75.4)	105 (83.3)	88 (84.6)	104 (87.4)	395	
	<b>130</b>	<b>126</b>	<b>104</b>	<b>119</b>	<b>479</b>	

**Table 3: Antibiogram and Susceptibility index of *S. ser. Typhi* from study area (%)**

	GOMBE			YOLA			Total (for the two areas)			SI
	S	I	R	S	I	R	S	I	R	
Amoxicillin	23(74)	0(0)	8(26)	32((60)	3(6)	18(34)	55(65)	3(4)	26(31)	2.12
Ampicillin	2(6)	0(0)	29(94)	6(11)	0(0)	47(89)	8(10)	0(0)	76(90)	0.11
Chloramphenicol	19(61)	0(0)	12(39)	27(51)	10(19)	16(30)	46(55)	10(0)	28(33)	1.64
Cotrimoxazole	6(19)	0(0)	25(81)	18(34)	0(0)	35(66)	24(29)	0(0)	60(71)	0.41
Tetracycline	11(35)	0(0)	20(65)	11(21)	3(6)	39(74)	22(26)	3(4)	59(70)	0.37
Ceftriaxone	20(65)	1(3)	10(32)	31(58)	9(17)	13(25)	51(61)	10(12)	23(27)	2.26
Ofloxacin	27(87)	2(6)	2(6)	33(62)	13(25)	7(13)	60(71)	15(18)	9(11)	6.45
Ciprofloxacin	6(19)	16(52)	10(32)	13(25)	21(40)	19(36)	19(23)	37(44)	29(35)	0.66
Nalidixic Acid	11(35)	4(13)	16(52)	17(32)	13(25)	23(43)	28(33)	17(20)	39(46)	0.72

**KEY**

S= Susceptible

I = Intermediate Susceptibility

R= Resistant

SI = Susceptibility index

**Table 4: Plasmid Carriage Profiles of *Salmonella ser. Typhi* isolated from the study Area**

Cure Resistant Profile	Yola	Gombi	Total
AmpCCo	9 (17)	11 (35)	20 (24)
AmpCTet	5 (9)	0(0)	5 (6)
AmpCCoCtx	1 (2)	0(0)	1 (1)
CoTet	2 (4)	0(0)	2 (2)
Ctx	6 (11)	4 (13)	10 (12)
AmpCTet	1 (2)	0(0)	1 (1)
AmpC	1 (2)	0(0)	1 (1)
AmpTetNaCo	0(0)	1 (3)	1 (1)
AmpCoTet	0(0)	1 (3)	1 (1)
AmpCoTetCtx	0(0)	1 (3)	1 (1)
Nil	28 (53)	13 (42)	41 (49)
	<b>53</b>	<b>31</b>	<b>84</b>

**KEY:**

Amp Ampicillin

C Chloramphenicol

Co Cotrimoxazole

Tet Tetracycline

Ctx Ceftriaxone

Na Nalidixic Acid

ESBL Extended Spectrum Beta lactamase

Values in parenthesis are percentages

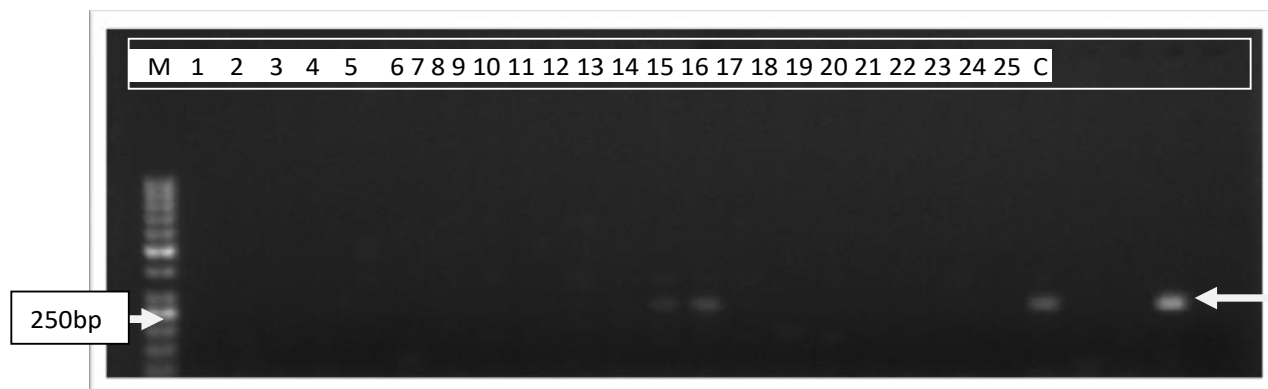


Plate 1:- Agarose gel electrophoresis pattern showing single PCR amplified products of *S. enterica* serovar Typhi *Blactx gene* in lanes number 12, 13, 21 and 24.  
Lane M :- DNA molecular marker (50- bp ladder), Lane C s:- Negative control

## DISCUSSION

The *Salmonella ser. Typhi* incidence from this study is higher in Yola (20.7%) compared to the 13.9% occurrence rates in Gombi. This goes on to buttress the fact that *Salmonella ser. Typhi* is endemic in the study since factors like low socioeconomic status, unhygienic practices, overcrowding, lack of proper sanitation and carrier status that have been linked to the persistence of *S. ser. Typhi* a population are prevalent in the study area.

More of the *Salmonella ser. Typhi* from this study was recovered from males than females. This finding is in agreement with previous studies in Adamawa Southern senatorial zone reported by Sale *et al.* (2017) where 20% of *Salmonella ser. Typhi* recovery was among males compared to 16.9% in female. It also agreed with earlier reports by Clark *et al.* (2010) who reported that 55% of adult typhoid cases were males in the United Kingdom. This result however is at variance with the report of Saleh *et al.*, (2014) who reported higher frequency of isolation from females (24%) than from males (9%). Higher occurrence in males could imply lower hygiene levels in males and the tendency to eat out often from unhygienic sources like road side food vendors. The fact however still remains that enteric fever occurrence has nothing to do with gender but low level of hygiene and eating habits (FAO, 2011).

From the result of antibacterial susceptibility test it was observed that the highest resistance (90%) was observed against ampicillin, followed by cotrimoxazole (71%) and tetracycline (70%). This finding is similar to the 83.3% ampicillin resistance among *S. ser. Typhi* isolates reported by Seljul *et al.* (2014) in Jos, Nigeria. Furthermore, it has been reported that 88% *S. ser. Typhi* isolates from Iraq are resistant to ampicillin (Rahman *et al.*, 2014) although lower

ampicillin resistance (15%) among *S. ser. Typhi* isolates have been reported in Egypt. This resistance pattern is not surprising as some of the factors that drive resistance such as the widespread self-prescription practice, incomplete dosage, availability of substandard or adulterated drugs in our markets exposes the organism to sub lethal doses, thereby acquiring resistance. In all, there were 57 resistance *Salmonella ser. Typhi* phenotypes out of the 84 isolates from the study area. The most common R type in the study area was AmpCCoTet. The isolates with the highest resistance in Yola was *Salmonella serovar Typhi YLA 21* demonstrating resistance to AmpCCoTetCtxCpNa and *Salmonella serovar Typhi GMB 6* showing resistance to AmxAMpCoTetCtxCpNa *in Vitro*. This implies that some of isolates are like superbugs demonstrating resistance *in vitro* to a wide range of antibiotics, thereby raising concerns on the treatment options available (Subramani and Vignesh, 2012; Riaz *et al.*, 2011).

Antibiotics susceptibility test of isolates revealed that 70% of isolates from Yola and Gombi were multi drug resistant with 49% and 55% being quinolone resistant respectively. These are lower than the 80% reported by Akinyemi *et al.* (2007) in Lagos, Nigeria. But higher than the 30.3% reported in Central Africa (Lunguya *et al.*, 2012); 66.7% in India (Akhtar *et al.*, 2015) and 22% reported by Menezes *et al.* (2011) in Pondicherry, India between 2005 and 2009.

Screening of the isolates for extended spectrum beta lactamase activity using the double disk synergy test revealed that *Salmonella enterica Typhi isolates GMB 1, 6, 13, 26* and *Salmonella ser. Typhi YLA 4, 11, 22, 31* and 42 produces the enzyme *in vitro*. This is not surprising as emergence of ESBL positive isolates have been reported and attributed to transfer of ESBL

genes to S ser. Typhi from non-Typhi strains (Rahman *et al.*, 2002). ESBLs are typically encoded on large; 80-kb to 300-kb plasmids that can be exchanged between bacterial species although a 23kb self-transmissible plasmid has been implicated in ESBL production in *Salmonella ser. Typhi* isolates by Akinyemi *et al.* (2015). In many cases, these plasmids also encode other antimicrobial resistance genes as observed in this study. Therefore, it is common for organisms expressing an ESBL to express co-resistances to aminoglycosides, trimethoprim-sulfamethoxazole, and tetracyclines (Jacoby and Medeiros, 1991).

Furthermore, PCR analysis revealed the presence of *Bla<sub>CTX</sub>* gene cluster in 4/25 (16%) of S. ser. Typhi isolates. This is higher than the 15.4% incidence reported in Kuwait but lower than the 50% reported in Iraq and 81 % in Lagos among S. ser. Typhi isolates (Rotimi *et al.*, 2008; Abdallah *et al.*, 2014 and Akinyemi *et al.*, 2015). The gene has been reported to be absent among S Typhi isolates in the United Arab

Emirate (Rotimi *et al.*, 2008). The detection of some strains with resistance to ceftriaxone as well as ability to produce the ESBL is an early warning sign indicating the need for more controlled use of this drug in the country and in the study area.

## CONCLUSION

The emergence of antimicrobial resistance among *Salmonella ser. Typhi* is a global problem that deserves attention. This is becoming more urgent due to the emergence of isolates demonstrating resistant to all the first line drugs as well as reduced susceptibility to quinolone and resistance to the third-generation cephalosporin ceftriaxone. The detection of some strains with resistance to ceftriaxone as well as ability to produce the ESBL is a wake-up call to all and sundry on the need to prescribed and use antibiotics on needs basis.

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