

Detection of *bla*_{CTX-M} gene on Resistant *Salmonella enterica* serovar. from a Hospital in Southeast Nigeria.

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Abstract

Typhoid fever appears to be a disease that has been associated with man since close to the first appearance of hominids. A total of twenty-five clinical isolates of *Salmonella enterica* serovars recovered between July-September, 2011 were investigated and screened using molecular methods for the presence of *bla*_{CTX-M} type gene and plasmids. Polymerase Chain Reaction analysis showed that the four of the *S. enterica* encoding the CTX-M type gene were mostly Extended Spectrum β -Lactamase producers which had no plasmids DNA. Phenotypic analysis reveals that the enzymes were active on Cefotaxime (64%) more than Cefotaxime (16%). Two folds serial dilution method reveals that Cefotaxime had increased Minimum Inhibitory Concentration (MIC) ranging from 1.56ug/ml- 12.5ug/ml depending on the isolates and the β -lactamase enzyme produced. The four *bla*_{CTX-M} gene detected on the adult Patients were from the General Out Patients Department (GOPD) and National Health Insurance Scheme (NHIS) section of the hospital in Eastern Nigeria.

INTRODUCTION

The emergence of resistance to antimicrobial agents within the salmonellae is a worldwide problem that has been associated with the use of antibiotics in livestock. Resistance to ceftriaxone and the fluoroquinolones, which are used to treat invasive salmonella infections, is rare in the United States^[1]. Typhoid fever is a systemic infection caused by *Salmonella Enterica* serotype typhi. This is a highly adapted human specific pathogen and possesses remarkable mechanism for persistence in host^[2]. Most of the disease burden occurs in developing countries due to poor sanitary conditions^[3]. Cefotaxime is a third-generation cephalosporin antibiotic. Like other third-generation cephalosporins, it has broad spectrum activity against Gram positive and Gram negative bacteria. In most cases, it is considered to be equivalent to ceftriaxone in terms of safety and efficacy. Cefotaxime is used for infections of the respiratory tract, skin, bones, joints, urogenital system, meningitis, and septicemia. The syn-configuration of the methoxyimino moiety confers stability to β -lactamase enzymes produced by many Gram-negative bacteria^[4]. Studies have also shown that resistance to broad-spectrum β -lactams is highly mediated by extended-spectrum β -lactamase (ESBL) enzymes, increasing the world health problem in clinical settings^[5,6,7]. According to^[7]. The plasmid-borne β -lactamases are also competent enough to hydrolyze β -lactam antibiotics, as well as the mechanism of resistance to β -lactam agents among gram-negative bacteria. Bacteria have acquired a variety of mechanisms to resist the action of antibiotics. The production of β -lactamases, enzymes that destroy penicillins and cephalosporins by hydrolyzing their β -lactam nucleus, is the most common mechanism of resistance^[8]. Cefotaximases (CTX-M) are class A β -lactamases that in general present higher levels of hydrolytic activity against cefotaxime than against ceftazidime. Ceftazidime MICs for organisms producing these enzymes are sometimes in the susceptible range. Many laboratories use ceftazidime resistance alone as an indicator of extended-spectrum β -lactamase production^[9]. More than 80

CTX-M enzymes are currently known. Despite their name, a few are more active on ceftazidime than cefotaxime. They have mainly been found in strains of *Salmonella enterica* serovar Typhimurium and *E. coli*, but have also been described in other species of Enterobacteriaceae and are the predominant ESBL type in parts of South America^[10]. Thus the use of molecular tool like Polymerase Chain Reaction (PCR) to detect this resistant gene in organism and to stop the spreading of this gene is essential, especially in this part of Africa. Since only phenotypic pattern is not discriminating enough to distinguish this organism as it depend on the growth of this organism especially

S. enterica serotype typhi. This study was aimed at using a fast, sensitive and specific molecular method such as PCR in detecting the prevalency of *bla*_{CTX-M} type gene, and Extended Spectrum β -lactamases (ESBL's) in Nigeria, most especially in the eastern part of Nigeria where antibiotics resistance to Cefotaxime and Ceftazidime are in the increase in the Hospitals.

MATERIALS AND METHODS

Bacteria Isolates;

A total of the 25 isolates of the Stool samples were collected from the several units in the hospital (July- September, 2011). Clinical human isolates of the *Salmonella enterica* used were obtained from the Routine Section of the Medical Microbiology Laboratory in the eastern part of Nigeria after basic culturing techniques and a reference strain negative of *bla*_{CTX-M} type gene.

The antibiotic- sensitivity screening was carried out by the multidisc agar diffusion method on 20ml molten muller Hilton Agar according to^[11] standard using these antibiotics disc (oxoid); Septrin 30 μ g, Chloramphenicol 30 μ g, Ciprofloxacin 10 μ g, Amoxicillin 30 μ g, Streptomycin 30 μ g, Levofloxacin 5 μ g, Ceftriaxone 30 μ g, Cefotaxime 30 μ g, Ceftazidime 30 μ g, Amoxicillin/Clavulanic acid 30 μ g. The tube dilution method of broth was used to determine the MIC of the test antibiotic against the clinical strains by the two fold serial broth dilution procedure according to^[11].

Serotyping using Slide Agglutination;

The slide agglutination antigen kit (Lab-Care Diagnostics, India) was used for this analysis. The cultured colony on a nutrient agar medium were isolated and inoculated on the grease free slide. A drop of physiological saline solution was introduced and the contents mixed using a mixing stick. The results were observed macroscopically after one minute, for auto-agglutinating strains after rocking the slide for 30 - 60 seconds. This procedure was repeated simultaneously with a drop of the antiserum (Lab-Care Diagnostics, India). The O antigen suspensions were tested first. If they were positive, then the monovalent O antisera belonging to this group were also performed. A positive result shows that the isolated bacterium possesses the antigen corresponding to the antiserum. The same procedures were subsequently carried out on the H antigen suspension^[12].

Plasmid Extraction;

The Alkaline Lysis method of⁽¹³⁾ was adopted for the extraction. 200µL of buffer 1A(400mM Tris, 20mM Na EDTA, Acetic acid to pH 8.0) was added to the cell pellets and vortexed. Then 400µL of the lysing solution (4% SDS (Sodium Dodocyl Sulphate) was added and the tubes were inverted 20 times at room temperature and 300µl ice cold buffer2B (3M Na Acetate, Acetic acid to pH 5.5) was added. Vortex the mixture and keep on ice for 30 minutes. Then centrifuge at 3,000xg for 15 mins. To the supernatant 700µL of chloroform was added and vortexed. Centrifuge at 3,000xg for 10 mins and transfer 500µL of the aqueous layer into a new tube. Then add 1ml of absolute ethanol and keep on ice for 1 hr. Centrifuge at 3,000xg for 30mins. then wash pellets with 70% ethanol, decant and dry pellets. 50µL of buffer 3C(10mM Tris, 2mM Na₂ EDTA, Acetic acid to pH 8.0.) was added and the kept for electrophoresis on ice.

Detection of ESBL's Positive Isolates using Double Disc Synergy Test (DDST);

The test inoculum (0.5 McFarland turbidity) was spread onto Mueller-Hinton agar (MHA;Oxoid, India) using a sterile cotton swab. A disc of augmentin (20 µg amoxycillin + 10 µg Clavulanic acid) was placed on the surface of MHA; then discs of ceftriaxone CRO (30 µg),Ceftazidime CAZ (30 µg) and Cefotaxime CTX (30 µg) were kept around it in such a way that each disc was at distance ranging between 16 and 20 mm from the augmentin disc (centre to centre). The plate was incubated at 37 °C overnight. Distances between the discs were required to be suitably adjusted for each strain in order to accurately detect the synergy. 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 Amoxillin/Clavulanic acid^[7].

Rapid Extraction, Quantification and Purity Test of the DNA;

1.5ml of an overnight broth culture was pipetted into eppendorf tubes and centrifuged at 5rpm or 5xg. The supernatant was discarded and sterile water was introduced into the tubes and the mixture is shaken vigorously by a vortexing machine and decanted. This process was repeated twice. Then the mixture was resuspended in 100µL sterile distilled water, vortexed and placed in a water block to boil for 10-20mins at 100°C. After boiling the mixture was then centrifuged for 10 mins and the purified chromosomal DNA pellets were transferred into a new tube and stored on ice. The spectrophotometer lens (Nano drop ND1000)

was cleaned with sterile distilled water and 0.2µL drop of the DNA sample is added on the lens, closed and then click the measure on the computer screen and the readings was observed. Purity level is known to be between 1.5 - 1.8 or 2µL (data not shown).

PCR Amplification and Agarose Gel Electrophoresis;

The purified DNA template in PCR eppendorf tubes (2.0µL DNA) was mixed with the Master mix ready to load (Solis Biodyne with 2.5mM MgCl₂) containing all the required contents as stated by the manufactures descriptions except water and the primers for the amplification. The mixture of primers (Biomers diaganostic), DNA and the Master mix was vortexed to mix and then centrifuged before introducing it into the PCR machine (Eppendorf-Germany). Then the machine is adjusted to annealing temperature and switch to start. After amplification has completed, the PCR products were then taken for electrophoresis (CBS.Scientific company Inc.) on the agarose gel at 80-100volts and finally viewed on the UV light for visible amplified image of the genes. A DNA-marker of 100base pairs was used.

The Polymerase Chain Reaction (PCR) were performed under the following conditions with the (Solis biodyne 5x FIREPol) Master mix Ready to load. The thermocycling condition used for *bla*_{CTX-M} were 30-35 cycles of 95°C for 30secs, 72°C for 1min.,66.2°C for 1 min., 72°C for 1min, 95°C for 30 sec.,(PCR timing 1.38- 2.58 hrs). The *bla*_{CTX-M} universal primer used were; *bla*_{CTX-M} - F(5'ATG TGC AGY ACC AGT AAR GTK ATG GC-3', 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 is to design to occupy for the ambiguity of sequence variation among the CTX-M types).

RESULTS AND DISCUSSION

Typhoid fever is a systemic infection caused by Salmonella Enterica serotype typhi. This is a highly adapted human specific pathogen and possesses remarkable mechanism for persistence in host^[2]. Most of the disease burden occurs in developing countries due to poor sanitary conditions^[3]. The results of the antibiotics screening using agar diffusion method were positively discovered in this study. Several reports on how to extract DNA rapidly from over night liquid culture as procedures for DNA extraction directly from a single colony as stated in^[14]. In this study we described and adopted a rapid method DNA extraction from directly from a single bacterial colony, which gave quality DNA for PCR in 15-20 mins. This protocol also gave a quality DNA products for PCR amplification and were tested for its quantity and purity which resulted in quality PCR fragments.

The report of the antibiotics resistant pattern of the twenty-five *S. enterica* isolated from stool as analysed in this study were as follows ; Amoxicillin/Clavulanic acid(92%), Amoxicillin (76%), Chloramphenicol (68%), Streptomycin (68%), Ceftazidime (64%), Co-trimoxazole (64%), Ciprofloxacin (24%), Cefotaxime (16%), Levofloxacin (12%), Ceftriaxone (12%). The Phenotypic expression of the antibiotics show that the β-lactamase enzyme produced were more active on Ceftazidime than on Cefotaxime as shown (Table 2). The results of the phenotypic analysis revealed that the β-lactamase were more active on the Ceftazidime, than on the Cefotaxime. This may be in support of the studies carried out by several authors revealing that resistance to broad-spectrum β-lactams is highly mediated by extended-spectrum β-lactamase (ESBL) enzymes, which has

Table 1. Epidemiological pattern of distribution of patient data and molecular analysis of the *bla_{ctx-M}* type gene of *S. enterica* detected from the hospital in the eastern part of Nigeria.

Code S/NO	Unit/ Dept.	Sex	Age Range (yrs)	B-lactamase/ CTX-M Production	Presence of Plasmid/Mwt.	No of antibiotics Resistant
SO3.	GOPD	F	Adult (18-above)	-ve/CTX-M	Nil	2
SO14.	GOPD	M	Adult (18-above)	-ve/CTX-M	Nil	9
O15.	GOPD	F	50	-ve/CTX-M	Nil	5
O24.	NHIS	M	Adult (18-above)	-ve/CTX-M	Nil	6

F= Female , M= Male , NHIS = National Health Insurance Scheme , GOPD= General Out Patient Department .Note: Adult (includes male and female between 18 and above, whose actual age was not determined).Nil= Negative.,CTX-M = Cefotaxime-M type enzyme, SO= Salmonella Owerri code.

Table 2. Phenotypic resistance pattern in relation to β - lactamase and ESBL'S production in *S. enterica* serovar.

CODE S/NO	NUMBER OF ANTIBIOTICS	RESISTANCE PHENOTYPIC PATTERN	B-LACTAMASE PRODUCTION	PRESENCE OF ESBL'S
SO3	2	CH, AMC	+VE	ESBL
SO9	7	SXT,CH,CPX,AM,STR,CAZ,AMC	+VE	ESBL
SO10.	6	SXT,CH,CPX,AM,CAZ,AMC	+VE	ESBL
SO11.	8	SXT,CH,CPX,AM,GEN,STR,CAZ,AMC	-VE	ESBL
SO12.	7	SXT,CH,CPX,AM,STR,CAZ,AMC	+VE	ESBL
SO13.	5	SXT,CH,AM,STR,AMC	+VE	ESBL
SO14.	9	SXT,CH,AM,STR,LEV,CRO,CTX,CAZ,AMC	+VE	ESBL
SO15.	5	SXT,CH,AM,STR,AMC	+VE	ESBL
SO19.	6	SXT,CH,CPX,AM,STR,AMC	+VE	ESBL
SO24.	6	AM,STR,CRO,CTX,CAZ,AMC	+VE	ESBL

SXT= Co-trimoxazole, CH= Chloramphenicol , CPX= Ciprofloxacin , AM= Amoxicillin, STR= Streptomycin , LEV= Levofloxacin , CRO= Ceftriaxone , CTX= Cefotaxime , CAZ= Ceftazidime, AMC= Amoxicillin/Clavulanic acid ,+ve = Positive, ESBL= Extended Spectrum Beta Lactamase., SO= Salmonella Owerri code.

Table 3: Minimum Inhibitory Concentration(MIC's) of some of the antibiotics tested on the bla_{CTX-M} positive *S. enterica* serovars.

CODE S/NO	ANTIGENIC PROPERTY	LEV (MIC)	CPX (MIC)	AMC (MIC)	CTX (MIC)	CRO (MIC)
SO3	<i>S. enterica</i> (O/H)	1.56	3.125	25.0	3.125	1.56
SO14	<i>S. enterica</i> (O/H)	25.0	3.125	25.0	6.25	6.25
SO15	<i>S. enterica</i> (O/H)	3.125	1.56	25.0	1.56	3.125
SO24	<i>S. enterica</i> (O/H)	1.56	1.56	25.0	12.5	12.5

KEY: SXT= Septrin 30ug, CH= Chloramphenicol 30ug, CPX= Ciprofloxacin 10ug, AM= Amoxicillin 30ug, STR= Streptomycin 30ug, LEV= Levofloxacin 5ug, CRO= Ceftriaxone 30ug, CTX= Cefotaxime 30ug, CAZ= Ceftazidime 30ug, AMC= Amoxicillin/Clavulanic acid 30ug., +ve = Positive, ESBL= Extended Spectrum Beta Lactamase., O = Somatic antigen, H= Flagellated antigen, SO= Salmonella code

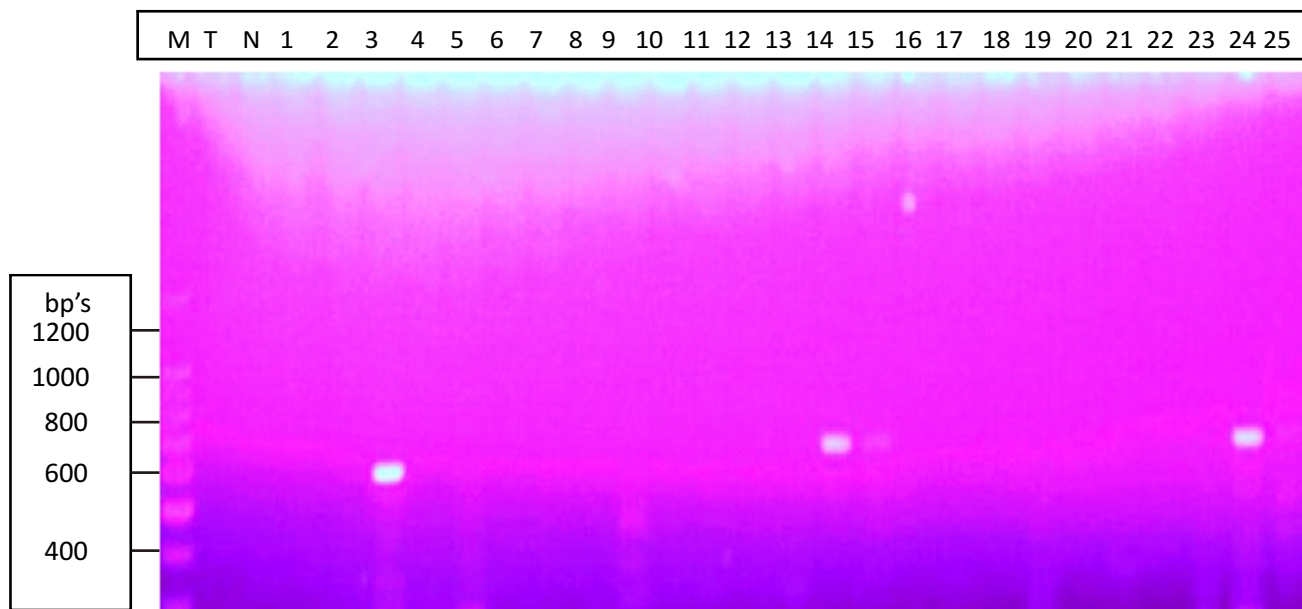


Fig.1: Agarose gel electrophoresis pattern showing single PCR amplification products of bla_{CTX-M} genes from *S. enterica* serovar Typhi. Lane 1- 25 shows isolates from FMC Owerri. The amplicons size (593bp's) on lane 3,14,15 and 24 shows positive bla_{CTX-M} genes present in the DNA of *S. enterica* serovar. typhi. Lane M shows DNA molecular marker(100- bp ladder). Lane T is the typed isolates of *S. typhi* ATCC14028 (negative for bla_{CTX-M}). Lane N contains only sterile water for control

increased the world health problem in clinical settings^[5,6,7].

The PCR amplification result shows that a total of four isolates were positive for bla_{CTX-M} gene type universal primer(two male and two female adult patients), but no plasmids were found encoding the gene of the *S. enterica* from the four patients(Fig.1). This report also shows that it is not all β -lactamase enzymes that causes resistant to antibiotics are mediated by plasmids. Analysis in these studies has shown that there is other mechanism of resistance like bla_{CTX-M} gene or Cefotaximases enzymes that mediates drug resistance especially in *S. enterica*. Also among ESBL family, the CTX-M type of enzyme is widely spread in many organisms in the world especially *S. enterica* serovars.

Furthermore, the Cefotaxime was seen to have increased MIC'S which ranges from 1.5 μ g/ml in isolates CODESO3 to isolates CODESO14 with MIC 6.25 μ g/ml. Other MIC of the Cefotaxime on isolate CODESO15 and CODESO24 were 1.56 μ g/ml and 12.5 μ g/ml (Table3). From Table 1., in this study, analysis reveals that both the *S. enterica* isolated from the female and the male adults having positive bla_{CTX-M} type gene all produced β -lactamase and were recovered from the General Out Patients Department (GOPD), except isolate CODESO24 which were isolated from a patient on National Health Insurance Scheme(NHIS). Though all the *S. enterica* positive to bla_{CTX-M} gene were resistance to various number of antibiotics, isolates CODESO3 was only resistant to 2 antibiotics, isolate CODESO14 was resistant to Nine of the

antibiotics tested, while isolate CODESO15 and CODESO24 were resistant to 5 and 6 antibiotics respectively. Also analysis revealed that out of the 25 *bla*_{CTX-M} tested, 10(40%) of the isolates produced β -lactamase enzyme while only 8(24%) were ESBL positive. Though not all of the ESBL positive *S. enterica* produced the *bla*_{CTX-M} type gene but all were possessing the somatic and flagellated antigenic properties. From the analysis the resistant to Ceftriaxone(12%) were less when compared to Cefotaxime (16%), while the resistance to Ceftazidime (64%) were higher. It should be noted that some organisms will not exhibit lactamase unless the enzyme has been induced by exposure to a beta-lactam antimicrobial and are called chromosomal Cephalosporinase, so some of the isolates which were resistant to some of the cephalosporins like Ceftazidime, Ceftriaxone and other combination of Amoxicillin/Clavulanic acid, may be due to this type of Cephalosporinase. Although the inhibitor-resistant β -lactamases are not ESBLs, they are often discussed with ESBLs because they are also derivatives of the classical TEM- or SHV-type enzymes according to [15]. Also, according to [9], Cefotaximases (CTX-M) are class A β -lactamases that in general present higher levels of hydrolytic activity against cefotaxime than against ceftazidime. But in some cases Ceftazidime MICs for organisms producing these enzymes are sometimes in the susceptible range. Also reports by [9] stated that many laboratories use ceftazidime resistance alone as an indicator of extended-spectrum β -lactamase production [9]. In this study as earlier stated Ceftazidime resistance was high, revealing the presence of this enzyme Phenotypically. Furthermore, analysis reveals that there were Multi drug Resistant (MDR) *S. enterica* based on the resistant number of antibiotics.

CONCLUSION

Many studies have demonstrated the use of molecular methods in the identification of specific bacterial genes that causes resistant in several organism especially, *S. enterica*. In this analysis the ability of PCR and the combination of other molecular method to achieve this purpose has been revealed. The identification of the *bla*_{CTX-M} type gene by PCR which was used to target the gene using primer sequences, has also shown that molecular methods is highly specific. The detection of ESBL's and B-lactamase as one of the mechanism of resistance used by *S. enterica* in this study has been revealed. Also, in this study, the first line of drugs such as Chloramphenicol, septrin and even the combination of Amoxicillin/ clavulanic acid used for the treatment of *S. enterica* was also seen to be highly resisted by this organisms due to the presence of this enzymes in the genes. Therefore care should be taken in the way most of this drugs are prescribed and used by patients in the community or in the hospitals. So proper diagnostic analysis is highly required and suggested for this purpose. Further molecular analysis is required to completely identify the actual CTX-M type of enzyme encoding the gene of this highly pathogenic organism.

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