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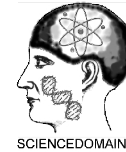

Emergence of Third Generation Cephalosporin Resistance and Typing by Randomly Amplified Polymorphic DNA (RAPD) among Clinical Salmonella Isolates from Lagos, Nigeria

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Authors' contributions

Author KOA conceived, designed, participated in the analysis and drafted the manuscript. Author BAI managed the analysis of the study and selected the primers. Author JAA participated in the literature search and the antimicrobial susceptibility testing: author OO participated in the literature search and antimicrobial susceptibility tests and plasmid analysis: author AKF contributed in search for literatures, ESBL analysis and susceptibility tests. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To investigate emergence of cephalosporin resistance and clonal relatedness among clinical Salmonella isolates recovered from patients in Lagos, Nigeria.
Study Design: It is an investigative study. A total of 300 patients who presented with various types of medical conditions at prominent referral public hospitals were recruited.
Place and Duration of Study: Department of Microbiology, Lagos State University, Ojo, Lagos and Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria from July 2011 to May 2012.
Methodology: Salmonella identification was done using standard methods. The isolates were subjected to antimicrobial susceptibility by disk diffusion method. The isolates were further screened for plasmid DNA and blaCTX-M carriage on plasmid using alkaline lysis and PCR methods. Clonal relatedness of the isolates was assessed by RAPD PCR using genomic DNA as template for three RAPD primers (784, 1254 and OPA4). The resulting

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RAPD types were determined by visualization and discrimination index was measured using a discriminating index calculator.

Results: Sixty three Salmonella isolates were recovered made up of five serovars. In all 49% of the isolates were resistant to cefuroxime, 46% to cefoxitin, 37% to cefriazone and 35% to ceftazidine. Interestingly 28(87.5%) of the 32 ESBL producing Salmonella isolates possessed at least one or more plasmids from five distinct sizes recorded 2.5; 4; 9; 15;23.5kb. Four distinct RAPD profiles were exhibited by the test strains. The total discriminatory power among the isolates was 0.77 (77%).

Conclusion: Third generation cephalosporin resistance involving blaCTX-M has emerged among clinical Salmonella isolates in Lagos. RAPD elicits potential as a cost-effective and time saving tool for local discrimination of clinical Salmonella isolates for epidemiological purposes.

Keywords: Salmonella; ESBL; drug resistance; emerging; RAPD; typing.

1. INTRODUCTION

The third generation cephalosporin antibiotics have been used over the years for the treatment of serious infections caused by *Salmonella* spp both in animal and human [1] Resistance to third generation cephalosporins due to acquisition and expression of extended spectrum β-lactamase (ESBL) enzymes among *Salmonella* isolates has currently emerged. The ESBL enzymes are usually plasmid mediated and are capable of hydrolyzing and inactivating a wide variety of beta-lactams, including third-generation cephalosporins [2,3]. ESBL producing organisms are reported to account for a significant proportion of intensive care infections. Although the growing incidence of ESBLs in *Salmonella* species and other microorganisms have been identified in numerous countries of Latin America, Africa, Europe and Asia [3,4], in Nigeria, information on ESBL producing *Salmonella enterica* serovars remains a conjecture. In recent times increasing treatment failures arising from the use of third generation cephalosporin among patients with *Salmonella*-associated diseases has been a major concern in most hospitals and clinics due to possible emergence of ESBLs producing strains of *Salmonellae*; an added constraint on already overburden health systems. Phenotypic, phage and molecular characterization methods in conjunction with traditional serology have been widely employed for typing *Salmonella* isolates. The last decade has witnessed the introduction and increased use of monoclonal antibodies, restriction enzymes, DNA probes and PCR techniques. Each of these tools has provided increased power and resolution [5]. Various molecular typing methods used for discrimination of *Salmonella* isolates, include pulse field gel electrophoresis (PFGE) analysis and multilocus sequence typing [6], IS200 typing, ribotyping, plasmid profiling, amplified fragment length polymorphism (AFLP) analysis and multilocus variable-number tandem-repeat analysis (MLVA). Some are relatively difficult to perform, slow and expensive whilst others have limited reproducibility and discriminatory powers [7]. Application of random amplified polymorphic DNA (RAPD) is more suitable than other techniques, because it does not require prior knowledge of target DNA and it uses short (9 to 10 bases) primers with a small amount of starting DNA [8]. RAPD has been used successfully for typing different organisms including gram negative bacteria. RAPD has been useful for epidemiological typing of *Salmonella* isolates from human outbreaks and from avian sources and for complementing serotyping and phage typing methods [5]. Therefore this study was initiated with a view to investigating the emerging resistant *Salmonella* isolates to third generation cephalosporin antibiotics and to identifying clonal relatedness among clinical *Salmonella* isolates using PCR-RAPD technique.

2. MATERIALS AND METHODS

2.1 Study Design

This is an investigative study**.** A total of 300 patients presented with various types of medical conditions at prominent referral public hospitals in Lagos from July 2011 to May 2012 were investigated. The patients were queried and diagnosed of having one and/or more of the following diseases; diarrhoea, malaria, HIV/AIDS, urinary tract infection (UTI), septicaemia, bacteraemia and pelvic inflammatory disease. The clinical samples collected under aseptic conditions from these patients were faeces, blood and urine. The samples were transported to the laboratory within 6 hours of collection and stored. It was stored under cool condition for further analysis.

2.2 Bacteriology

The samples were processed in the laboratory by standard methods [8]. The media used for the cultivation are MacConkey agar (Oxoid, UK), Blood agar (Oxoid, UK), desoxycholate citratrate (Oxoid, UK) agar and *Salmonella-Shigella* agar (Oxoid, UK), Colonies were first subjected to biochemical tests as described by Cowan and Steel [9]. The commercially-available identification system API 20E (bio-Mérieux, France) was used. Colonies considered to be *Salmonella* spp. were further tested for somatic (O) and flagella (H) antigens with polyvalent antisera (Wellcome Diagnostic, UK).

2.3 Antimicrobial Susceptibility Testing

All *Salmonella* isolates were investigated for their *in vitro* susceptibilities to antibiotics: cefotaxime 30 ug; ceftaxidime 30 ug; ceftriazone 30 ug; cefoxitin 30 ug; imipenem 30 ug; lavofloxacin 10 ug; and augmentin 30 ug (20 ug amoxicillin/10 ug clavulanic acid).by disk diffusion as described by Clinical and Laboratory Standard Institute (CLSI; formerly NCCLS) guidelines [10].

2.4 Extended Spectrum Beta-lactamase Assay

All the isolates that exhibited reduced susceptibility and/or resistance to third generation cephalosporin were screened for ESBL production, using Double Disc Synergy Test Method (DDST) [11]. DDST was done to determine synergy between a disc of 30 µg augmentin (20 µg amoxicillin and 10 µg clavulanic acid) and 30 µg of each third generation antibiotics (3GC) i.e ceftazidime (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), and cefoxitin (30 µg), (Oxoid, U.K). For quality control, *Klebsiella pneumoniae* ATCC 700603 was used as the positive control and *E. coli* ATCC 25922 as the negative control in each test batch.

2.5 Plasmid DNA Extraction

Plasmid extraction was performed on all the isolates positive for ESBL production. It was done by using TENS – Mini Prep as described by Chen Zhou et al. [12].

2.6 Genomic DNA Preparation

Genomic DNA was extracted by boiling method as described by Freschi et al. [13]. The supernatant was then transferred to a new tube and diluted 1:5 v/v with sterile water to obtain a final DNA concentration of 50 – 100 ng/uL before use as template DNA for RAPD PCR.

2.7 Detection of Β-lactam Resistance Genes- DNA Aplification by PCR Technique

The extracted plasmid DNA was used as template for the detection of ESBL *bla* genes. The 25 ul PCR reaction mixture contained 10 pmol of each forward and reverse primer pair for the *bla* genes, 200 micromol of deoxynucleotide triphosphate, 1U of Taq DNA polymerase and 1 ul of plasmid DNA solution.. The PCR programme consisted of an initial denaturation step of 5 minutes at 94ºC, a 30 cycle period (each consisting of 60s at 94ºC, 30s at 58ºC, and 30s at 72ºC), then a final extension step of 5 minutes at 72ºC. The primer nucleotide sequences for blaCTX-M OS-5 5′-TTA TCT CCC TGT TAG CCA CC-3′ and OS-6 5′-GAT TTG CTG ATT TCG CTC GG-3' were those reported for *Salmonella* sp. by Rotimi et al. [14].

2.8 RAPD Reaction

For RAPD PCR analysis the parameters in each PCR reaction was optimized in order to maximize discriminatory power of the reaction for typing *Salmonella* isolates. The three set of primers used include: 784; 5' GCG GAA ATA G 3', 1254; 5' CCA CAG CCA A 3'and OPA4 primer sequence 5'AAT CGG GCT G 3' (Biomers, Germany). In each PCR reaction, the DNA template solution (2uL) was mixed with 18uL of PCR reaction mixture to make 20uL total volume reaction comprising dNTPs (200uM each), $MgCl_2$ (3 mM), OPA4 RAPD primer (30 picomole), x1 PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), Taq polymerase (1.5 Units) and sterile de-ionized water. The reaction for 784 and 1254 was x1 buffer, 2 mM $MgCl_{2,}$ 200uM of each dNTPs, 40 picomoles of primer and 1U Taq polymerase. The amplification reaction occurred in a thermocycler (200 ATC, Biorad USA) using the following amplification parameters: 94ºC for 6 minutes followed by 40 cycles of 94ºC for 30s, 35ºC for 30s and 72ºC for 1 min. Finally incubation at 72ºC for 5 minutes was performed.

2.9 Agarose Gel Electrophoresis

Electrophoresis of the plasmid DNA, blaCTX-M and RAPD PCR products was carried out on 0.8% and 1.5% agarose gel at 100 V for 1 h respectively. Bands were viewed under ultraviolet light for photo-documentation. DNA bands were sized by extrapolation using 1kb and 100 bp DNA molecular weight markers.

2.10 Plasmid DNA Curing Method

The plasmid curing was carried out by a method described by Scheleif and Wenbiuk [15] with Acridine Orange treatment.

2.11 Conjugation Experiment

Conjugation experiments were performed as described by Karmakar et al. [16] using *E. coli* V517 strain from Nigerian Institute of Medical Research (NIMR) as recipient.

3. RESULTS AND DISCUSSION

Of the 300 samples evaluated, 63 (21%) *Salmonella* isolates were recovered, of which 44 (%) isolates were from faeces, 10 (%) from blood and 9 (%) from urine. The 63 *Salmonella* isolates comprised of 17 (27%) *S. typhimurium*, 4 (6.4%) of *S. typhi*, 28 (44.4%) of *S. enteritidis*, 5 (7.6%), 9 (14.3%) of *S. paratyphi* and *S. cholerasuis* (Table 1). The isolation of non-typhoidal *Salmonella* in this study further confirmed the reports from Nigeria that *S. enteritidis* and *S. typhimurium* are frequently associated with cases of gastroenteritis [17]. We found in this study, 2 strains of *S enteritidis* and a strain of *S. typhimurium* from faeces of HIV patients. The *Salmonella* co-infection with HIV might add to possible depletion in immunity in these patients. Also, six *Salmonella* isolates were recovered from malaria parasiteamia two of which were from blood samples. Previous workers elsewhere had documented an association between falciparum malaria and *Salmonella*-associated bacteraemia in malaria-endemic regions [17].

The results of antimicrobial susceptibility tests revealed that 49% of the isolates were resistant to cefuroxime, 46% to cefoxitin, 37% to cefriazone and 35% to ceftazidine. The resistance recorded in *Salmonella* isolates to these antibiotics is worrisome and quite revealing; this is because the third generation antibiotics are currently the drug of choice for severe complicated typhoidal and non-typhoidal salmonellosis in Nigeria. The isolates showed high susceptibility to leavofloxacin (95%) and imipenem (90%) (Table 2).

All the isolates were screened for ESBL production, 32(52.1%) of the isolates were ESBL positive. and 53.1% of ESBL positive isolates produced blaCTX-M genes. ESBL production has been detected in *Salmonella enterica* strains of different serovars in several countries, like Italy, France, and Nepal [18,19]. However, to the best of our knowledge, this is the first report to describe an infection caused by an ESBL-producing *Salmonella* isolates in Lagos, Nigeria. Interestingly 28(87.5%) of the 32 ESBL producing *Salmonella* isolates possessed at least one or more plasmids from five distinct sizes recorded 2.5; 4; 9; 15;23.5kb. Seventeen (52.1%) of the 32 ESBL positive *Salmonella* isolates produced blaCTX-M gene (Fig. 1) and were found to possess at least a plasmid DNA. At least one or more strains of each *Salmonella* serotype possessed plasmid and blaCTX-M gene except *S. paratyphi* which did not exhibit blaCTX-M genes in this study (Table 3). Previous work had shown that blaCTX-$_2$ and blaCTX-M$_{-3}$ are the most resistance genes for ESBL producing *Salmonella* strains that are plasmid-borne and can be transmitted among bacterial organisms of the same or different species, resulting in wide-spread resistance [20]. In this study the prevalence of blaCTX-M genes among *Salmonella* isolates was 26.9%, while prevalence among ESBL-producing strains of *Salmonella* was 53.3%. Ramazanzadeh et al. [21], reported an incidence of 31.7% of blaCTX-M genes and among all *Salmonella* spp. 3 (2.3%) ESBL-producing isolates carried the blaCTX-M genes, while in a recent study in Kuwait and the United Arab Emirates, 3.4% of *Salmonella* isolates were CTX producers [14]. The emergence of an ESBL in *Salmonella serotypes* constitutes a new challenge. This may be explained by the exchange of mobile genetic elements, such as plasmids through conjugation experiment, between enteric bacteria and was selected for by extended-spectrum cephalosporin. Since ESBL genes are nearly always located on mobile genetic elements such as plasmid DNA and transpooson [19], further rapid spread among *Salmonella* strains is to be expected. Therefore this study showed evidence of production of an ESBL, CTX-M, in *Salmonella spp* due to the presence of a blaCTX-*M* gene (Fig. 2). This is a worrisome finding with potential serious clinical implications, since the dissemination of this resistance trait will further hamper the therapeutic possibilities for the treatment of *Salmonella*-associated diseases in our environment.

The results of 63 *Salmonella* isolates subjected to PCR RAPD typing using three primers 784, 1254 and OPA4 revealed that only OPA4 primer discriminated *Salmonella* isolates. The Primers 784 and 1254 did not produce any discrimination amongst the *Salmonella* isolates and obviously no typing was possible. This result is in consonance with study conducted in Lagos Nigeria by Smith et al. [22] that primers 784 and 1254 did not discriminate amongst the *Salmonella* isolates. The implication of this is that RAPD primers 784 and 1254 are not useful for typing *Salmonella* isolates in our locality. However, this study was in contrast to the report of Quintanes et al. [23] which recorded highest discrimination power amongst clinical *Salmonella* isolates with primers 784 and 1254 in Brazil. It has been reported that RAPD technique is laboratory-dependent and needs carefully developed protocols and selection of appropriate primers to be reproducible [24]. In this study the RAPD OPA4 primer was found useful for typing *Salmonella* isolates as eight RAPD types were observed among 63 *Salmonella* isolates. Specifically, two distinct RAPD types A&D were observed among the 4 strains of *S. Typhi.* Similarly, two RAPD types B &G for *S. typhimurium* and RAPD types E&F for *S. enteritidis*. No discrimination was observed among strains of *S. cholerasuis* (C) and S. *paratyphi* (F) as shown in Table 3.The uniqueness of the RAPD patterns in this study was that the isolates were distinctive with regard to biochemical profiles and serotypes. The data obtained from amplification products by strains were used to estimate genetic similarity among the isolates on the basis of shared amplified product. There was no discrimination observed for *S. paratyphi* and *S. cholerasuis* which indicated that there was no similarity between the sources of these isolates. Interestingly, all the strains of *S. paratyphi* were from feacal samples and *S. cholerasuis* from the blood samples. It has been document that results generated by the RAPD should be interpreted with caution as occurrence of random genetic events, such as point mutation, insertion and deletion of DNA can alter the RAPD patterns [22], an assertion that could not be ruled out in this study. Nevertheless, the results of single RAPD OPA4 primer used in this study has shown degree of clonality among *Salmonella* isolates in our environment.

Table 1. *Salmonella* serotypes associated with diarrhea and extra-intestinal infections from 3 clinical samples

Organism	Faeces						Blood							Urine							n
	Dia	Mal	Hiv	Uti	Sep	P1	Dia	Mal	Hiv	Uti	Sep	P1	Bac	Dia	Mal	Hiv	Uti	Sep	P1	Bac	
S. enteritidis	16	2	2	1			2	1			2			1			1				28
S. typhimurium	6		1					1			2		1				5		1		17
S. typhi	2	1													1						4
S. cholerasuis	8						1														9
S. paratyphi	5																				5
Total	37 0	3	3	1	0		3 1	2	0	0	4	0		1		1	6		1		63

Key: n – Total number of isolates
Dia- Diarrhea Mal-Malaria; Hiv- HIV-AIDS
Uti-Urinary Tract Infection; Sep- Septicaemia
Pl- Pelvic Inflammatory disease; Bac- Bacteremia

Table 2. Antibiotics resistance pattern of *Salmonella* isolates (n=63)

Organism	No isolated				Antibiotics used				
		CTX	CXM	CRO	CAZ	AMC	FOX	IPM	LEV
S. typhi	4	1	1	2	2	-	2	-	1
S. paratyphi	5	3	2	3	1	3	3	-	-
S. cholerasuis	9	1	3	2	6	-	2	2	-
S. enteritidis	28	6	14	8	9	8	10	3	-
S. typhimurium	17	3	11	8	4	3	12	1	2
Total	63	14	31	23	22	14	29	6	3
Percentage		22%	49%	37%	35%	22%	46%	10%	5%

Key: CTX Cefotaxime, CXM Cefuroxime, CRO Ceftriazone, CAZ Ceftazidime, AMC augmentin, FOX Cefoxitin, IPM Imipenem LEV, Levofloxacin

Table 3. Plasmid analysis, ESBL production and RAPD-PCR profile in *Salmonella* isolates

***Salmonella* serotypes**	**No**	***RAPD PCR Profile**				**@RARD Types**	**Plasmid number**				**Rage of plasmid size in kb.**	**bla ctx-M(+ve)**
		1	**2**	**3**	**4**		**0**	**1**	**2**	**3**		
S. typhi	4	2	2	0	0	A,D	0	2	1	1	15-23.5	2
S. paratyphi	5	0	5	0	0	F	3	1	0	1	15-23.5	0
S. typhimurium	17	5	5	7	0	B,G	11	3	3	0	4.0-23.5	4
S. enteritidis	28	3	8	10	6	E,F	17	6	4	1	2.5-23.5	6
S. cholerasuis	9	-	3	3	-	C	4	1	3	1	2.5-23.5	5
Total	63	10	23	20	6		35	13	11	4	2.5-23.5	17 (27)

*Data are presented in figures with percentage of isolates positive for blaCTX-M indicated in parenthesis. *discrimination index was calculated using a discrimination power calculator http://insilico.ehu.es/mini_tools/discriminatory_power/*

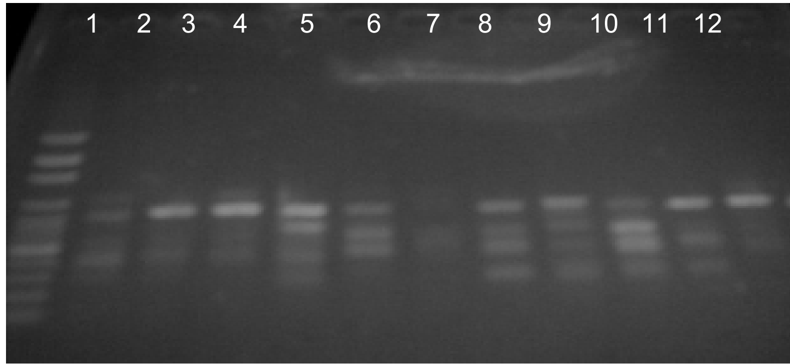

Fig. 1. Randomly amplified polymorphic DNA profiles of typhoidal and non-typhoidal *Salmonella* isolates recovered from patients in Lagos.

Lane 1 = 100 bp DNA ladder marker (10,000 – 100 bp); Lane 2 = S. typhi 004; Lane 3 = S. typhimurium 038; Lane 4 = S. cholerasius 034; Lane 5 = S. typhi 013; Lane 6 = S. enteritidis 022; Lane 7 = S. enteritidis 025; Lane 8 = S. paratyphi 02; Lanes 9 – 11; S. enteritidis 018,027 029; Lane 12 = S. typhimurium 042.

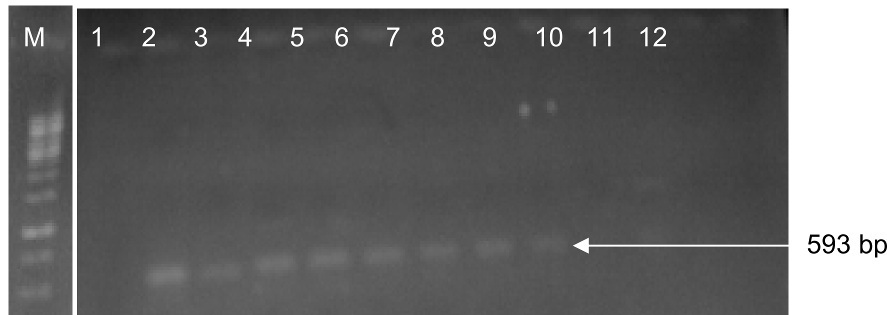

Fig. 2. Agarose gel electrophoresis of blaCTX-M gene recovered from some of the ESBL phenotypically positive *Salmonella* isolates.

Lane 1 = 10 kb DNA ladder marker; Lanes 1 – 4 = S. enteritidis strains; Lanes 5 & 6 = S. cholerasius strains; Lanes 7 & 8 = S. typhimurium strains; Lanes 9 – 12 = S. typhi strains.

4. CONCLUSION

The study revealed emerging plasmid-mediated ESBL-producing *Salmonella* strains carrying the blaCTX-M genes to third generation cephalosporin. This development if unchecked, would compromise the efficacies of these antibiotics in future, thereby posing a threat to public health. The study identified a single RAPD primer OPA4 that could be used to provide moderate discrimination amongst clinical isolates of *Salmonella*. This may be useful for small laboratories dealing with typing and surveillance studies where the cost of using combined primers could not be afforded. Generally, third generation cephalosporin resistance involving blaCTX-M has emerged among clinical *Salmonella* isolates in Lagos. RAPD elicits potential as a cost-effective and time saving tool for local discrimination of clinical *Salmonella* isolates for epidemiological purposes.

CONSENT

The consents of the patients recruited for this were sought.

ETHICAL APPROVAL

This study was approved by the ethical committee of the Lagos State University, Ojo, and Lagos.

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COMPETING INTERESTS

Authors have declared that there are no conflicting interests.

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