

Emerging third generation cephalosporin resistance and single RAPD primer for typing clinical *Salmonella* isolates from Lagos, Nigeria

Abstract

Aim: To study investigated the antimicrobial resistant status and RAPD typing of *Salmonella* isolates from patients

Study design: A total of 300 patients who presented with various types of medical conditions at prominent referral public hospitals were recruited for the study. The patients were diagnosed of having one and/or more of the following diseases; diarrhea, urinary tract infection (UTI), septicemia, bacteriaemia and pelvic inflammatory disease. Faeces, blood and urine were the samples processed.

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: Three hundred were evaluated for bacteriological analysis using standard methods. The isolates were subjected to antimicrobial susceptibility testing using the following antibiotics: cefotaxime 30ug; ceftaxidime 30 ug; ceftriazone 30ug; cefoxitin 30 ug; imipenem 30 ug; laevofloxacin 10 ug; and augmentin 30 ug (20 ug amoxicillin/10 ug clavulanic acid). The isolates were screened for plasmid DNA and RAPD PC R method was applied to differentiate the isolates.

Results: Sixty three *Salmonella* isolates were recovered made up of five serovars. About 25% of the isolates were resistant to cefuroxime, ceftazidime and ceftriazone. Specifically, 28 isolates possessed plasmids with size ranging from 2.5-23.5 kb. Four distinct RAPD profiles were exhibited by the test strains. The total discriminatory power among the isolates was 0.77 (77%).

Conclusion: Emerging plasmid mediated drug resistant *Salmonella* serotypes to third generation cephalosporins were revealed. The study identified a single RAPD primer OPA4 that could be used to provide a simple and rapid moderate discrimination amongst clinical isolates of *Salmonella*.

Key words: Salmonella, Drug resistance, emerging, RAPD, Typing

Introduction

The third generation cephalosporin antibiotics have been used over the years for treatment of serious infections caused by *Salmonella* 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 had 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 organisms 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 concerns 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. 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), multilocus sequence typing (6), IS200 typing, ribotyping, plasmid profiling, amplified fragment length polymorphism (AFLP) analysis 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 (7). Therefore this study was initiated with a view to investigating the emerging resistant *Salmonella* isolates to third generation

cephalosporin antibiotics and to identifying faster, easier and cheaper method for prompt detection and diagnosis of *Salmonella* isolates from clinical samples.

Materials and Methods

Study design: A total of 300 patients who presented with various types of medical conditions at prominent referral public hospitals in Lagos from July 2011 to May 2012. The patients were 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. It was stored under cool condition for further analysis.

Bacteriology: The samples were processed in the laboratory by standard methods. The media used for the cultivation are MacConkey (MC) agar, Blood agar, desoxycholate citrate (DC) agar and *Salmonella Shigella* agar (SS), Colonies were first subjected to biochemical tests as described by Cowan and Steel (8). 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).

Antimicrobial Susceptibility testing: All *Salmonella* isolates were investigated for their *in vitro* susceptibilities to antibiotics by disk diffusion as described by Clinical and Laboratory Standard Institute (CLSI; formerly NCCLS) guidelines (9)

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) (10). 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,

85 *Klebsiella pneumoniae* ATCC 700603 was used as the positive control and *E. coli* ATCC
86 25922 as the negative control in each test batch.

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88 **Plasmid DNA Extraction:** Plasmid extraction was performed on all the isolates positive for
89 ESBL production. It was done by using TENS – Mini Prep as described by Chen Zhou *et al.*
90 (11).

91 **Genomic DNA Preparation;** Genomic DNA was extracted by boiling method as described by
92 Freschi *et al.*, (12). The supernatant was then transferred to a new tube and diluted 1:5 v/v with
93 sterile water to obtain a final DNA concentration of 50 – 100 ng/uL before use as template DNA
94 for RAPD PCR.

95 **Detection of B-lactam resistance genes- DNA Amplification by PCR Technique;**

96 The extracted plasmid DNA was used as template for the detection of ESBL bla genes. The 25 ul
97 PCR reaction mixture contained 10 pmol of each forward and reverse primer pair for the bla
98 genes, 200 micromol of deoxynucleotide triphosphate, 1U of Taq DNA polymerase and 1 ul of
99 plasmid DNA solution.. The PCR programme consisted of an initial denaturation step of 5
100 minutes at 94°C, a 30 cycle period (each consisting of 60s at 94°C, 30s at 58°C, and 30s at 72°C),
101 then a final extension step of 5 minutes at 72°C. The primer nucleotide sequences for blaCTX-M
102 were those reported for *Salmonella* sp. by Rotimi et al (13); OS-5 5'-TTA TCT CCC TGT TAG
103 CCA CC-3' and OS-6 5'-GAT TTG CTG ATT TCG CTC GG-3'

104 **RAPD reaction:** For RAPD PCR analysis the parameters in each PCR reaction was optimized in
105 order to maximize discriminatory power of the reaction for typing *Salmonella* isolates. The three
106 set of primers used include: 784; 5' GCG GAA ATA G 3', 1254; 5' CCA CAG CCA A 3'and
107 OPA4 primer sequence 5'AAT CGG GCT G 3' (Biomers, Germany). In each PCR reaction, the
108 DNA template solution (2uL) was mixed with 18uL of PCR reaction mixture to make 20uL total
109 volume reaction comprising dNTPs (200uM each), MgCl₂ (3 mM), OPA4 RAPD primer (30
110 picomole), x1 PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), Taq polymerase (1.5 Units)
111 and sterile de-ionized water. The reaction for 784 and 1254 was x1 buffer, 2 mM MgCl₂, 200uM
112 of each dNTPs, 40 picomoles of primer and 1U Taq polymerase. The amplification reaction
113 occurred in a thermocycler (200 ATC, Biorad USA) using the following amplification

parameters: 94°C for 6 minutes followed by 40 cycles of 94°C for 30s, 35°C for 30s and 72°C for 1 min. Finally incubation at 72°C for 5 minutes was performed. The amplification products were detected by gel electrophoresis in 2% agarose gel at 70 V for 60 minutes in 1 x TAE buffer.

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.

Plasmid DNA curing method; The plasmid curing was carried out by a method described by Silhavry *et al.* (14) with Acridine Orange treatment.

Conjugation experiment; Conjugation experiments were performed as described by Yukata *et al* and Wang *et al.*, (15) using *E. coli* V517 strain from Nigerian Institute of Medical Research (NIMR) as recipient.

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Results and Discussion

143 Of the 300 samples evaluated, 63 (21%) *Salmonella* isolates were recovered, of which 44 (%)
 144 isolates were from faeces, 10 (%) from blood and 9 (%) from urine. The 63 *Salmonella* isolates
 145 comprised of 17 (27%) *S. Typhimurium*, 4 (6.4%) of *S. Typhi*, 28 (44.4%) of *S. Enteritidis*, 5
 146 (7.6%), 9 (14.3%) of *S. Paratyphi* and *S. Cholerasuis* (Table 1) . The isolation of non-typhoidal
 147 *Salmonella* in this study further confirmed the reports from Nigeria that *S. Enteritidis* and *S*
 148 *Typhimurium* are frequently associated with cases of gastroenteritis (16).

149 The results of antimicrobial susceptibility tests revealed that 49% of the isolates were resistant to
 150 cefuroxime, 46% to cefoxitin, 37% to ceftriazone and 35% to ceftazidime. The resistance recorded
 151 in *Salmonella* isolates to these antibiotics is worrisome and quite revealing; this is because the
 152 third generation antibiotics are currently drug of choice for severe complicated typhoidal and
 153 non-typhoidal *Salmonellosis* in Nigeria. The isolates showed high susceptibility to leavofloxacin
 154 (95%) and imipenem (90%) (Table 2). All the isolates were screened for the presence of ESBL It
 155 was observed that 52.12% were positive and 53.1% of ESBL positive isolates produced bla-ctx
 156 gene. Interestingly 28(87.5%) of the 32 ESBL producing *Salmonella* isolates possessed at least
 157 one or more plasmids raging in size from 2.5 to 23.5 kb. Seventeen (52.1%) of the 32 ESBL
 158 positive *Salmonella* isolates produce bla-ctx gene (Figure 1) and were found to possess at least a
 159 plasmid DNA. Study had shown that *bla*_{CMY-2} and *bla*_{CTX-M-3} are the most resistance genes for
 160 ESBL producing *Salmonella* strains that are plasmid-borne and can be transmitted among
 161 bacterial organisms of the same or different species, resulting in wide-spread resistance (17). In
 162 this study the prevalence of bla ctx genes among *Salmonella* isolates is 26.9%, while prevalence
 163 among ESBL-producing strains of *Salmonella* is 53.3%. Ramazanzadeh *et al*,(18), reported an
 164 incidence of 31.7% of *bla*_{CTX} genes and among all *Salmonella* spp. (n =129), the 3 (2.3%)
 165 ESBL-producing isolates carried the *bla*_{CTX} genes. while in a recent study in Kuwait and the
 166 United Arab Emirates, 3.4% of *Salmonella* isolates were CTX producers (13). The high

prevalence of ESBL-producing isolates carrying the *bla*_{CTX} genes calls for concern as the efficacies of third generation cephalosporin antibiotics are now threatened

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 bla-ctx genes. 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 produce bla-ctx gene (Figure 1) and were found to possess at least a plasmid DNA.. At least one or more strains of each *Salmonella* serotype possessed plasmid and bla-ctx gene except *S. Paratyphi* which did not exhibit bla-ctx genes in this study (Table 3).

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 use of Primers 784 and 1254 did not produce any discrimination amongst the *Salmonella* isolates, as most of the isolates did not exhibit any amplification band even after three PCR repeats. This result is in consonance with study conducted in Lagos Nigeria by Smith *et al.* (19) that primers 784 and 1254 did not discriminate amongst the *Salmonella* isolates. However, this study was in contrast to the reports of Quintanes *et al.*(20) which recorded highest discrimination power amongst clinical *Salmonella* isolates with primers 784 and 1254 in Brazil respectively. It is obvious therefore that none of these two primers is suitable for typing *Salmonella* isolates from Nigeria. It has been reported that RAPD technique is laboratory-dependent and needs carefully developed protocols to be reproducible (21)

Eight RAPD types were found among 63 *Salmonella* isolates with RAPD OPA4 primer. There was 50% discriminatory power observed among the 4 strains of *S. Typhi* with two distinct RAPD types A&D, 38% of discrimination with RAPD types B &G for *S. Typhimurium* and 49% with RAPD types E&F for *S. Enteritidis*. This primer produced a discriminatory index of 0.77 for the five *Salmonella* serotypes. The uniqueness of the RAPD patterns in this study was that the isolates were distinctive with regard to biochemical profiles and serotyping. 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. Cholerasuis* and *S. Paratyphi* were isolated from feacal samples, and only *S. Cholerasuis* was from the blood samples.

Conclusion

The study revealed emerging plasmid -mediated ESBL-producing *Salmonella* strains carrying the *bla*_{CTX} genes to third generation cephalosporins. This development if unchecked, would compromise the efficacies of these antibiotics in future, thereby posing a threat to public health. Also RAPD-PCR was found to be a good technique for discriminating clinical isolates of *Salmonella* from our environment. The study identified a single RAPD primer OPA4 that could be used to provide moderate discrimination amongst clinical isolates of *Salmonella*. This is considered cheaper or less economical for small laboratories dealing with diagnosis and surveillance particularly in developing countries where the cost of using combined primers could not be afforded.

Ethical Committee: This study was approved by the ethical committee of the Lagos State University, Ojo, Lagos.

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Key: n – Total number of isolates

SENT-S. Enteritidis, STP – S. Typhimurium, S. Typhi, SCHOL-S. Cholerasesuis,

SPA-S. Paratyphi Dia- Diarrhea

Mal-Malaria Hiv-HIV-AIDS

Uti-Urinary Tract Infection Sep-Septicaemia

Pel Pelvic Inflammatory disease Bac-Bacteremia

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	
SENT	16 2 2 1	2 1 2	1 1	28
STP	6 1	1 2 1	5 1	17
ST	2 1		1	4
SCHOL	8	1		9
SPA	5			5
Total	37 3 3 1 0 0	3 2 0 0 4 0 1	1 1 6 1	63

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

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

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Salmonella serotypes	No	RAPD PCR Profile				RARD Types	Discriminating index	Plasmid number				Rage of plasmid size in Kleb.	ESBL _ve	Blax CTL (+ve)
		1	2	3	4			0	1	2	3			
S. Typhi	4	2	2	0	0	A,D	0.5	0	2	1	1	15-23.5	3	2
S. Paratyphi	5	0	5	0	0	F	MD	3	1	0	1	15-23.5	2	0
S. Typhriumurin	17	5	5	7	0	B,G	0.38	11	3	3	0	4.0-23.5	9	4
S. Enteritidis	28	3	8	10	6	E,F	0.49	17	6	4	1	2.5-23.5	13	6
S. Cholerasisuis	9	-	3	3	-	C	N.D	4	1	3	1	2.5-23.5	5	5
Total	63	10	23	20	6		0.77	35	13	11	4	2.5-23.5	32	17

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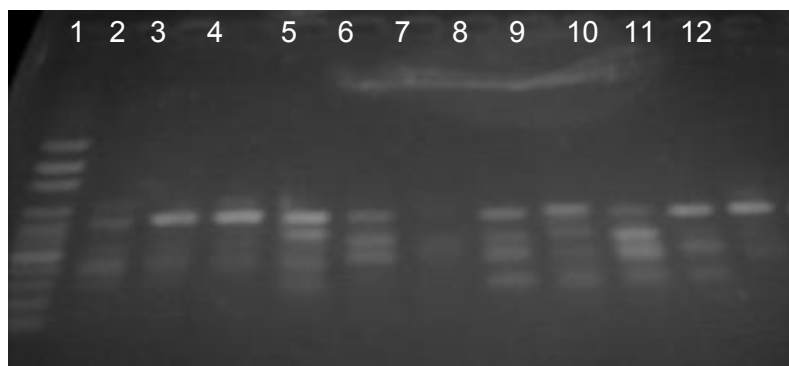


Figure 1. Randomly amplified polymorphic DNA profiles of typhoidal and non-typhoidal *Salmonella* isolates recovered from clinically ill Nigerian patients in Lagos. Lane 1 = 100 bp DNA ladder marker (10,000 – 100 bp); Lane 2 = *S. typhi*004; Lane 3 = *S. typhimurium* 038; Lane 4 = *S. choleraesuis*034; 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, 029; Lane 12 = *S. typhimurium* 042.



Figure 2: Agarose gel electrophoresis of *bla*ctx 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. choleraesuis* strains; Lanes 7 & 8 = *S. typhimurium* strains; Lanes 9 – 12 = *S. typhi* strains.