



## Random Amplified Polymorphic DNA Analysis and Extended Spectrum Beta-Lactamase Profile of Multi-drug Resistant *Salmonella* Isolates

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### ABSTRACT

Seventy-two (72) *Salmonella* organisms isolated from unrelated patients were evaluated for antimicrobial sensitivity in the disk diffusion method against 15 antibiotics. The resistant isolates were also screened for ESBL production in the double disc diffusion test. The molecular epidemiology of twenty (20) multidrug resistant isolates out of the total 72 isolates was also investigated using RAPD-PCR with OPC2, OPC4 and OPA9 primers to ascertain their genetic variability. The results obtained from the susceptibility assay of the seventy-two (72) *Salmonella* isolates obtained in this study were as follows: 65.2% of the isolates were susceptible to ofloxacin while the resistance rates for augmentin, gentamicin, ciprofloxacin and chloramphenicol were 72.2% (16.7% intermediate susceptible), 29% (11.1% intermediate susceptible), 20.8% (30.5% intermediate susceptible) and 56.9% (34.7% intermediate susceptible), respectively. It was observed from the results of this study that among the total *Salmonella* isolates, 90.3% were multiple drug resistance (MDR) with the pattern of resistance cut across 3 to 13 different antibacterial agents. In this study 32(44.4%) of the isolates were confirmed ESBL producers by the amoxicillin-clavulanic acid test. Of the different primer sets used for RAPD analysis, amplification using OPA9 resulted in three (2) RAPD types for the six isolates. The strains were grouped into two main clusters of three clones, denominated as isolates 1 to 14. Three (50.0%) of the amplified isolates showed close clonality with 2 bands. Similarly, 2 (33.3%) other isolates exhibited close clonality with a total number of 8 bands. Only one isolate was grouped separately with a distinct band pattern. The result of this study indicates that multidrug resistant *Salmonella* species with distinct genetic disparity are in circulation in the study area.

**Keywords-** : *Salmonella*, RAPD, Resistance, Primers, Antibiotics, Clonality

## INTRODUCTION

*Salmonella enterica* Serovar Typhi, a member of the enterobacteriaceae occasionally result in life threatening infections which culminate in proportionate morbidity and mortality. This diverse group of organisms has developed mechanisms which allow them to thrive in a wide array of environments and across different hosts (1). The onset of Infection follows the ingestion of food or water that has been contaminated such that *Salmonella* reach the intestinal epithelium with resultant abdominal discomfort. Upon ingestion of this organism into the intestinal epithelium, the infection has been reported to spread with subsequent dissemination within the body (2). It has been estimated that there are more than 21.7 million illnesses associated with Gram-negative enteric pathogens worldwide due to diarrhea and enteric fever each year (3).

*Salmonella* spp. are responsible for several outbreaks of human gastroenteritis and enteric fever all over the world. This bacteria species is widely distributed in different animal species (particularly poultry) that are used as food and represents a serious public health problem.

In many developing nations, including Nigeria, enteric fever is often diagnosed presumptively on the outcome of Widal serological test and antibiotic treatment is administered empirically without knowledge about the susceptibility of the infecting pathogen. The resultant low positive predictive value (PPV) associated with widal test and the variations in antibiotic susceptibility profile of *S. Typhi* strains means that a lot of patients are likely to receive inappropriate antibiotic therapy in

the absence of culture and antibiotic susceptibility testing (4).

All over the world, there has been rising cases of *Salmonella* isolates that are resistant to multiple antibiotics (5). Mounting surveillance to monitor drug resistance pattern among these group of organisms will provide clinicians with vital clues on the right therapeutic options to be adopted against individual cases. It could render reliable information regarding administration of drug to a particular locality on herd basis. Hence, it is imperative that judicious use of antibiotics in the treatment and prophylaxis, after *in vitro* testing, be practiced to sustain the efficacy of the antibiotics in controlling *Salmonella* infections on long-term basis.

The variation in susceptibility of *Salmonella* species to prominent antibiotics used for treatment of typhoid infections as well as the recurrence of this infection in treated individuals necessitates a proper elucidation of the mechanisms responsible for this trending observation. Therefore, molecular typing studies with the strains of this organism isolated from human sources, may improve the understanding of their epidemiology.

Several genotypic and phenotypic techniques including biotyping, ribotyping, random amplification of polymorphic DNA (RAPD-PCR), enterobacterial repetitive intergenic consensus (ERIC-PCR) and pulsed field gel electrophoresis (PFGE) have been used previously in separate studies by Ktari *et al.* (6) and Bouallègue-Godet *et al.* (7) to subtype *Salmonella* strains.

Typing of bacterial isolates using molecular tools allows for the examination of two or more isolates to determine their relatedness

(8). The choice of Random Amplified Polymorphic DNA (RAPD) over other molecular methods is because it utilizes short (9 to 10 bases) primers, requires small amount of DNA and it doesn't require prior knowledge of the target DNA (9). Molecular typing methods such as the RAPD-PCR method have been used for the differentiation and characterization of *Salmonella* and to trace the clonality of strains (10). RAPD has been useful for epidemiological typing of *Salmonella* isolates from human outbreaks. This study was aimed at investigating the genetic relatedness of multi-drug resistant *Salmonella* isolates obtained from human faecal sample.

#### MATERIALS AND METHODS

Faecal samples were collected from patients/students attending four different clinics all in Umuahia the capital of Abia State, Nigeria. Each patient gave an informed consent prior to sample collection. Samples were processed within a maximum of 2h after collection. The stool specimens were inoculated into Selenite Fecal broth (Titan Biotech Ltd, India) for enrichment and incubated at 37°C for 18-24h and subsequently plated on Salmonella-Shigella agar (Titan Biotech Ltd, India) for primary isolation. The culture plates were incubated at 37°C for 24h and observed for growth through the formation of colonies. After incubation for 24h, colonies were purified and maintained on Nutrient agar slants. All the bacterial isolates were identified using cultural, morphological and biochemical tests.

#### Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the disk diffusion method of the National Committee for Clinical Laboratory Standard (11) on Mueller Hinton

agar (Hardy Diagnostics, USA). The discs (Abtek Biologicals Ltd) used are: Cotrimoxazole (COT; 25µg), Amoxicillin (AMX; 25µg), Chloramphenicol (CHL; 30µg), Cefotaxime (CTX; 30µg), Ciprofloxacin (CPR; 5µg), Nitrofurantoin (NIT; 300µg), Ofloxacin (OFL; 10µg), Cloxacillin (CXC; 25µg), Erythromycin (ERY; 5µg), Streptomycin (STR; 30µg), Augmentin (AUG; 30µg), Gentamicin (GEN; 10µg), Ceftriaxone (CRO; 30µg), Ceftazidime (CAZ; 30µg) and Cefuroxime (CXM; 30µg). Discrete colonies from a 24hrs nutrient agar plate were suspended into sterile nutrient broth in a tube to achieve a bacterial suspension equivalent to 0.5McFarland turbidity Standard. A cotton swab was dipped into the bacterial suspension and the swab pressed on the side of the tube to drain excess fluid. The entire surface of the agar plate was then inoculated with the same swab of inoculum, rotating the plate to ensure confluent growth of the bacteria. This was done alongside with *Escherichia coli* ATCC 25922 which served as a control. The antimicrobial susceptibility disks were placed onto the dried, inoculated agar plate with a sterile forceps and the plates incubated in an inverted position for 16–18hrs at 37°C.

#### Test for ESBL

Resistant isolates obtained by disc diffusion assay was selected for further detection of ESBL by double disc synergy test (DDST) as described by Kaur *et al.*, (12). A lawn culture of the resistant organisms was made on a Mueller-Hinton agar plate (Hardy Diagnostics, USA). Antibiotics Discs (Oxoid, United Kingdom) containing amoxicillin-clavulanate (30µg) was placed in the centre of the plate. Discs (Oxoid, United Kingdom) of ceftriaxone (30µg), Cefotaxime (30µg) and ceftazidime (30µg)

were placed 15mm and 20mm apart respectively, centre to centre to that of the amoxicillin-clavulanate disc and plates incubated at 37°C for 18-24hrs. A clear extension of the edge of the inhibition zone of any of the antibiotics towards the disc containing amoxicillin-clavulanate was considered as positive for ESBL production.

### Genomic DNA Extraction and RAPD Assay

DNA was extracted from the isolates using standard protocol provided in the extraction kit (Zymo Research, California, USA). Extracted DNA was resolved through Tris Acetic acid EDTA (TAE) agarose gel electrophoresis prepared in a concentration of 0.8% of molecular biology grade agarose (Bioline, UK) in 1× TAE buffer. The gel was mixed with 10µl Ethidium bromide. Ten (10µl) of DNA template were mixed with 2µl of loading dye and visualized under UV light using UV transilluminator. Molecular weight standard marker (Fermentas) was used to get comparative detection of band sizes.

The amplification reaction was performed in a thermocycler (A & E Lab, United Kingdom) using OPC2, OPC4 and OPA9 primers. The PCR cycle started with an initial denaturation step at 94°C for 10min. This was followed by 30 cycles of denaturation at 94°C for 1min, annealing at 58°C for 1min and extension at 72°C for 1min, and a final extension at 72°C for 5min that was then followed by cooling to 4°C. Ten (10µl) of the samples were run on a 1% agarose gel at 90V for 30min in order to verify amplification. The bands generated from the RAPD assay was analysed using the PyElph (version 1.4) software which constructed the dendrogram as shown in fig.3 below.

### RESULT AND DISCUSSION

Seventy-two (72) *Salmonella* isolates were obtained from 135 fecal specimens studied in this investigation. Susceptibility test results by means of agar disc diffusion were as follows: 65.2% of the isolates were susceptible to ofloxacin; resistance rates for augmentin, gentamicin, ciprofloxacin and chloramphenicol were 72.2% (16.7% intermediate susceptible), 29% (11.1% intermediate susceptible), 20.8% (30.5% intermediate susceptible) and 56.9% (34.7% intermediate susceptible), respectively (Table 1). The decrease in the susceptibility of the *Salmonella* isolates to ofloxacin and ciprofloxacin as obtained from this investigation has been reported in other parts of Nigeria (9).

It was observed from the results of this study that among the total *Salmonella* isolates, 90.3% were multiple drug resistance (MDR) with the pattern of resistance cut across 3 to 13 different antibacterial agents. Increased MDR has been reported among *Salmonella* isolates in many countries including Iran (13, 14). A previous study in Nigeria by Akinyemi *et al.*, (15) had reported a multi drug resistance rate of 61.0%, other authors from Ethiopia (16) reported a multi drug resistance rate of 83.3%. The result of this study however revealed a comparatively high proportion of multidrug resistance with a frequency of 90.3%. The prevalence of multi-drug resistance reported in this work, limits the effective treatment of human *Salmonella* infection with commonly used antibiotics.

Results for ESBL production by the isolates in the double disk diffusion test showed that 32(44.4%) of the isolates were ESBL positive (table 2). This finding was comparable with earlier report of Menezes *et al.* (17), where 48% (10/21) of the non-typhoidal salmonellae studied exhibited

ESBL phenotype and Ndiba (23) who recorded a 37.5% prevalence rate. Detection of several strains of ESBL producing *Salmonella enterica* isolates have been reported across countries like France, Nepal and Italy (18). The increased usage broad spectrum cephalosporins could be a contributing factor for the high rate of selection of extended spectrum beta lactamase production by the isolates. It has however been demonstrated by Tersia *et al.* (19) that the resistance genes blaCTX-2 and blaCTX-M-3, are responsible for transmitting resistance amongst ESBL producing *Salmonella* strains which culminates in widespread drug resistance. In the study by Akinyemi *et al.* (9) the prevalence of blaCTX-M genes among *Salmonella* isolates was 26.9%, while prevalence among ESBL producing strains of *Salmonella* was 53.3%. ESBLs have thus evolved as a means of disseminating drug resistance.

Fingerprinting of twenty (20) of the multidrug resistant (MDR) *Salmonella* isolates (labeled as 1-20) with OPA primers resulted in the amplification of only six isolates which generated variable patterns amongst the multidrug resistant isolates that may indicate dissemination and circulation of different clones of *Salmonella* (fig. 2).

The isolates were grouped into two RAPD profiles of three clones, represented as A, B, and C. Isolates in Lane 3, 9 and 10 exhibited close clonality with 2 bands each. Similarly, isolates in Lane 2 and Lane 7 showed close clonality with a total number of 8bands. Only one representative (Lane 6), was

grouped separately with a distinct band pattern. Although a total of 20 MDR isolates were subjected to RAPD-PCR, only six isolates were significantly amplified (fig.2) after repeated runs with several OPA primer sets. The limitation of low discriminatory power and non-reproducibility as has been reported by other authors could have been the reason behind non-amplification of the rest of the isolates (20, 9). The result of this investigation is also supported by data obtained by Gurakan *et al.* (21) who reported that random amplified polymorphic DNA analysis has the potential to detect polymorphism throughout the entire genome as compared with other techniques, also Smith *et al.* (20) showed that the RAPD-PCR would be useful for epidemiological typing of the *Salmonella* spp in Nigeria and Yaqoob *et al.* (22) reported that RAPD analysis was applied for molecular characterization of *Salmonella enteritidis* strains.

The dendrogram tree constructed from RAPD data showed a common lineage among all six amplified isolates which indicated the majority of *Salmonella* isolates studied are descendents of the same microorganism that has differed little and is responsible for the transfer of resistance to antibiotics in the human population.

Our study indicates the circulation of genetically distinct clones. In conclusion, the *Salmonella* strains studied were genetically distinct, suggesting that a prevalent subtype may have been causing infection and transmitting resistance.

**Table 1: Antimicrobial Susceptibility Pattern of the *Salmonella* isolates**

Antimicrobial Agents	No. (%) Sensitive	No. (%) Resistant	No. (%) Intermediate
Gentamicin (10µg)	43 (59.7)	21 (29.2)	8 (11.1)
Chloramphenicol (30µg)	6 (8.33)	41 (56.9)	25 (34.7)
Ofloxacin (30µg)	47 (65.2)	12 (16.7)	13 (18.0)
Augmentin (30µg)	8 (11.1)	52 (72.2)	12 (16.7)

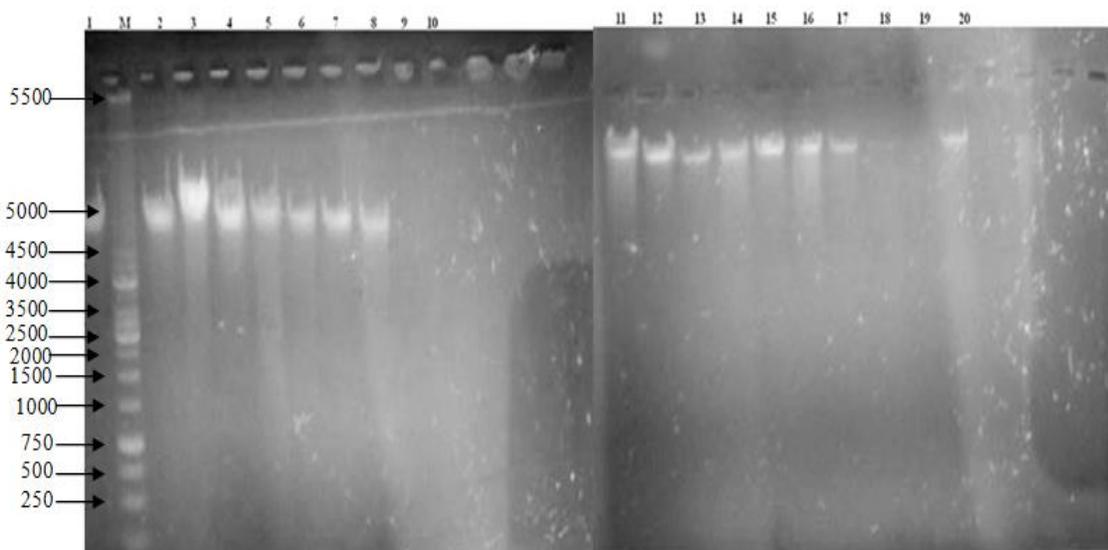
Ciprofloxacin (5µg)	35 (48.6)	15 (20.8)	22 (30.5)
Amoxicillin (25µg)	3 (4.17)	62 (86.1)	7 (5.18)
Cefuroxime (30µg)	2 (2.78)	51 (70.8)	19 (26.4)
Erythromycin (5µg)	0 (0.00)	72 (100.0)	0 (0.00)
Cotrimoxazole (25µg)	11 (15.2)	52 (72.2)	9 (12.5)
Cefotaxime (30µg)	2 (2.78)	59 (81.9)	11 (15.2)
Streptomycin (25µg)	8 (11.1)	47 (65.2)	17 (23.6)
Cloxacillin (25µg)	0 (0.00)	72 (100.0)	0 (0.00)
Ceftazidime (30µg)	6 (8.33)	47 (65.2)	19 (26.4)
Nitrofurantoin (100µg)	36 (50.0)	34 (47.2)	2 (2.78)
Ceftriaxone (30µg)	8 (11.1)	49 (68.0)	15 (20.8)

\*the susceptibility, intermediate and resistance states were interpreted according to Clinical Laboratory Standards Institute, CLSI (2015).

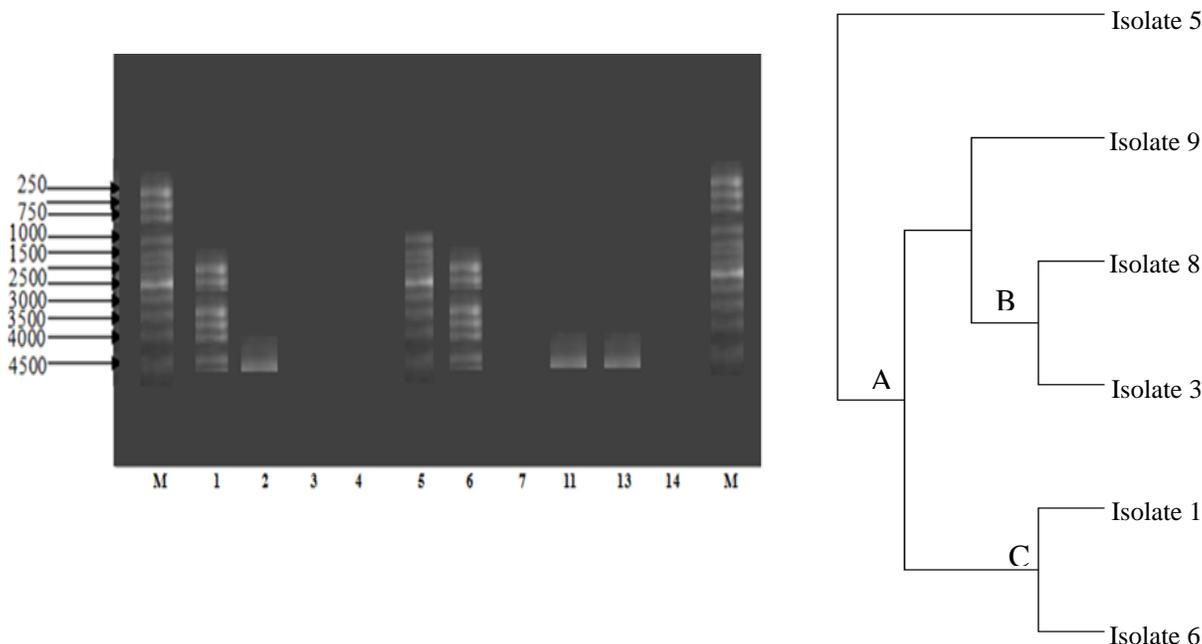
**Table 2: Multi-Drug Resistance and ESBL Profile of the Isolates**

	No. (%) MDR	No. (%) ESBL positive
<b>Male (n=30)</b>	27 (37.5)	12 (16.7)
<b>Female (n=42)</b>	38 (52.8)	20 (27.8)
<b>Total (n=72)</b>	65 (90.3)	32 (44.4)

MDR = Multi-Drug Resistance (The number of antibacterial agents varied from 3 to 13 antimicrobial agents); ESBL= extended spectrum beta-lactamases; ESBL positive was determined when each of the third generation cephalosporin had a >5mm zone of inhibition alone than when used together with Amoxicillin-Clavulanate determined when each of the third generation cephalosporin had a >5mm zone of inhibition alone than when used together with Amoxicillin-Clavulanate.



**Fig. 1: Agarose Gel Electrophoresis of genomic DNA extracted from the multi-drug Resistant *Salmonella* isolates (M= Marker; 1-20= isolates)**



**Fig 2a: RAPD Profile of the isolates using OPA 9 Primers. M= Marker; 1-14= amplified isolates. Fig 2b: Dendrogram obtained from the amplicons after RAPD assay.**

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