



Fluoroquinolones Resistance and Plasmid Profile Patterns of Typhoidal *Salmonella* Serovars Isolated from Human Faecal Samples in Tertiary Hospitals in South-East, Nigeria

Ezinwanne N. Ezeibe^{1*}, Ebele B. Onuigbo¹, Chinenye N. Ugwu¹, Dinebari P. Berebon¹, Thaddeus H. Gugu¹, Chinekwa S. Nwagwu², Anthony A. Attama^{2*}

¹Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria

²Department of Pharmaceutics University of Nigeria, Nsukka, Enugu State, Nigeria

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ABSTRACT

Antimicrobial resistance associated with salmonellosis is a major global public health challenge. This study investigated the resistance and plasmid profile patterns of typhoidal *Salmonella* serovars isolated from human faecal samples to commonly used fluoroquinolones. A total of 300 faecal samples were collected from three tertiary hospitals in South-East Nigeria. The isolates were characterized using standard phenotypic, molecular and antibiogram studies. Antibiogram studies were evaluated using ciprofloxacin and ofloxacin discs. Data were analysed with descriptive statistics, t-test and 2-way analysis of variance. Out of 300 faecal samples, (129) 43% yielded *Salmonella* spp and (62) 48% were confirmed to be typhoidal *Salmonella* serovars. The antibiogram study on the 62 typhoidal *Salmonella* serovars showed that (22) 35 % and (4) 7 % were sensitive, (27) 44 % and (28) 45 % were intermediate, (13) 21 % and (30) 48 % were resistant to ciprofloxacin and ofloxacin respectively. The plasmid profile revealed the presence of DNA with molecular weight ranging from 10 to 37 kb. The isolates had higher molecular weight than the control that was used for the plasmid profiling. On exposure to a curing agent (acridine orange), all the resistant isolates became sensitive. This study showed that the incidence of resistant typhoidal *Salmonella* to most commonly used fluoroquinolones is high in South-East Nigeria and this could be as a result of plasmid resistant DNA. It also indicated that the indiscriminate use of ciprofloxacin and ofloxacin within the South-East region of Nigeria may increase the typhoidal *Salmonella* resistance.

Keywords: Human faecal samples; typhoidal salmonella; antimicrobial resistance; plasmid profile pattern; fluoroquinolones; tertiary hospitals.

Introduction

Salmonella spp. is an important zoonotic pathogen that is majorly implicated in food borne related illnesses and typhoid fever.¹ Typhoid fever is a life-threatening infection caused by typhoidal *Salmonella* serovars. It is rated the 8th commonest infection globally.²⁻⁴ Globally, WHO (2018) estimates that 11-20 million people develop typhoid fever annually and about 128,000-161,000 people die because of the disease.⁵ Despite available antibiotic therapy for typhoid fever, mortality rate because of the fever remains high.⁶⁻⁷ Meanwhile, the mortality rate due to typhoid fever varies from region to region. It remains a significant health burden, especially in low- and middle-income countries.⁸ Hence, the fever is more endemic in African and Asian countries than in Western Europe and North America.^{2, 9} For instance, out of the 2.9 per 100,000 mortality rate as a result of typhoid fever in Western Sub-Saharan Africa, Nigeria alone has 2.6 per 100,000 people.

*Corresponding author. E mail: ezinwanne.ezeibe@unn.edu.ng;

anthony.attama@unn.edu.ng

Tel: +2348036207844

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In fact, the annual mortality rate per 100,000 people from typhoid fever increased by 7.0 percent since 1990, resulting to an average of 0.3 percent.¹⁰

Over the decades traditional first line drugs were used for the treatment of typhoidal *Salmonella* until it became resistant to the first-line drugs.¹¹ Consequently, fluoroquinolones and extended spectrum cephalosporins were introduced as antibiotics of choice in the treatment of multiple drug resistant typhoidal *Salmonella* in the 1980s.¹²⁻¹⁶ The use of fluoroquinolones for the treatment of typhoid fever became widespread in Africa in the early 2000.^{3, 14, 17}

Although the different classes of fluoroquinolones are used for the treatment of typhoidal *Salmonella*,¹⁷⁻¹⁸ ciprofloxacin and ofloxacin are the commonly used first-line fluoroquinolones for the treatment of typhoidal *Salmonella*, especially in Nigeria.¹⁹⁻²⁰ In 2015 for instance, out of 32.5 million prescriptions for oral fluoroquinolones, 20.3 million were ciprofloxacin.²¹ Despite the prevalence of antibiotics for the treatment of salmonellosis, the infections remain a major cause of death in Africa.²² In Nigeria typhoid fever and malaria are estimated to constitute about 50 % of all hospitalisation.¹⁰ The persistence of typhoid fever in Nigeria is associated with misuse and abuse of antibiotics as well as the emergence of fluoroquinolone resistant *Salmonella*.²³ Fluoroquinolone resistance can either be chromosomally mediated or plasmid-mediated.³³ Plasmids have majorly contributed in the spread of antibiotic resistant genes in microbial population.²³

Although previous studies have examined the importance of fluoroquinolones in treatment of *Salmonellosis*, they often neglect the antimicrobial resistance and plasmid profile pattern of typhoidal *Salmonella* to commonly used fluoroquinolones in South East, Nigeria.²⁴⁻²⁵ Despite the prevalence of typhoidal *Salmonella* in South

East Nigeria, including Enugu, Anambra, Ebonyi, Abia and Imo states,^{20,26} studies often neglect this region and focus on other regions, especially the South West, Nigeria.^{15,22-23} The aim of this study was to investigate the resistance and plasmid profile patterns of typhoidal *Salmonella* serovars isolated from human faecal samples in tertiary hospitals in South-East, Nigeria to commonly used fluoroquinolones.

Materials and Methods

Clinical samples

Human faecal samples were collected from the laboratories of three tertiary hospitals in South East Nigeria, including Alex Ekwueme Federal Teaching Hospital, Abakaliki, Ebonyi State, University of Nigeria Teaching Hospital, Ituku-Ozalla, Enugu State and Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State.

Chemicals and reagents

Crystal violet, 70 % ethanol, Lugol iodine, methyl red indicator, TE buffers (Merck, Germany), safranin, immersion oil, agar rose gel, ethyl alcohol, D-glucose, lactose powder (BDH Chemicals Ltd Poole, England), phenol red, distilled water, hydrogen peroxide, kovac's reagent, 1XTBE, 5XTBE, ethidium bromide.

Culture media

The culture media used include, nutrient agar, nutrient broth, Mueller-Hinton agar, *Salmonella*-*Shigella* Agar (SSA) (Oxoid, England), Selenite F broth (Biotech Laboratories Ltd, UK), acridine orange and agarose gel.

Antibiotic disc

Ciprofloxacin disc (Oxoid, England), ofloxacin disc (Oxoid England).

Collection and Transportation of Samples

Ethical approval (No. DOR/UNN/18/0022A) was obtained from the Research Ethics Committee of Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka before the commencement of the study. A total of 300 faecal samples were obtained from patients of different age and sex, attending three teaching hospitals within South-East Nigeria from 2017 to 2018. These patients had an erythrocyte sedimentation rate (ESR) above 15 mm/hr (Westergren) and presented with fever without diarrhoea or confirmed typhoid infections. The faecal samples were collected in batches of 30 each from the laboratories of the three tertiary hospitals in a sterile sample container. These faecal samples were put in ice-cold chambers and transported to the Department of Pharmaceutical Microbiology and Biotechnology Laboratory, University of Nigeria, Nsukka within 3 to 4 h of collection for isolation process and stored in the refrigerator (4°C) for further studies.

Isolation, purification and identification of bacterial isolates of salmonella

Selenite F nutrient broth, *Salmonella*-*Shigella* agar and nutrient agar slants were aseptically prepared using the manufacturers guidelines prior to the isolation of *Salmonella* spp. Cultivation and isolation of *Salmonella* spp were carried out on the clinical samples (faeces) by inoculation into the freshly prepared Selenite F broth, and incubated at 37 °C for 24 h, and subsequently sub-cultured by streaking on each of the *Salmonella*-*shigella* agar plates and incubated at 37 °C for 24 h. The plates were then examined carefully for the presence of characteristic colonies of *Salmonella* spp. The isolates identified as *Salmonella* spp from the *Salmonella*-*shigella* agar (SSA) were purified by further streaking on SSA. Identification and confirmation was done by specific biochemical Tests and microscopy (Gram staining).

Susceptibility studies

Procedure for sensitivity analysis

The commercially available antibiotics that were used for the study were ciprofloxacin (CP) (5µg) and ofloxacin (OF) (5µg).

The antimicrobial sensitivity of the test strains of the two antibiotics was done using the Kirby-Bauer disk diffusion method using Mueller-Hinton agar as earlier described.^{1,4,15,22} The plates were incubated at

37°C for 24 h. According to the Clinical Laboratory Standard Institute (CLSI) (2018), the diameters of the inhibition zones were measured planimetrically in millimetre at 24 h.²⁹ The organism was interpreted as sensitive if the inhibition zone diameter is ≥ 31, intermediate if the inhibition zone diameter is between 21 - 30 mm and resistant if the inhibition zone diameter is ≤ 20 for both ciprofloxacin and ofloxacin discs.

Plasmid profile analysis

The procedure for plasmid profile analysis was done for the 10 most highly resistant *Salmonella* isolates indicated by their low inhibition zone diameters (IZDs) according to clinical laboratory standard institute (CLSI). The alkaline plasmid DNA extraction method of Birnboim and Doly was used in this procedure.

Electrophoresis of DNA samples and visualization of the DNA

Gel electrophoresis was done to visualize the DNA. Briefly, with the aid of a micro-pipette, 10 µl of the standard was carefully loaded in the first well after which 10 µl of the test samples were slowly loaded respectively into their respective gel wells. After 45 min, the gel was gently removed from the casting tray and placed carefully on UV trans-illuminator where the DNA plasmids were visualized under the UV light. The molecular weights of the plasmids were thereafter determined from the electrophoregram.

Plasmid curing

The plasmids were cured by treatment with acridine orange according to the method adopted by Adeyemo and Onilude.²⁷ This was done to determine if the resistance were conferred by the plasmids. Inocula of 100 to 300 cfu/ml were incorporated into different concentrations of acridine orange nutrient broth (pH 7.6). This was then incubated at 37 °C for 24 h. Cultures with the highest concentration of acridine orange in which there was a visible growth was further diluted and sub-cultured on Mueller-Hinton agar plates, the antibiotic discs were gently and firmly placed on agar plates above and was incubated at 37°C for 24 h and the inhibition zone diameters were appropriately measured and recorded.

Statistical analysis

Descriptive and inferential statistics were used for analysis in the study. While frequency tables, pie chart and bar charts were used to analyze the colony characterization and biochemical analysis; t-test and 2-way analysis of variance (ANOVA) were used to analyze the sensitivity studies.

Results and Discussion

Isolation and colony characterization

The result shows that out of 300 faecal samples collected from laboratories in South East Nigeria, 129 (43 %) yielded *Salmonella* species because of the blackish colonies clearly seen. This yield was very high and may have been due to the low level of hygiene observed by the population in this region.³⁴ The isolation of *Salmonella* strains from faecal samples indicates that the *Salmonella* spp are prevalent and endemic in South East Nigeria.³⁴ Thus, Akinyemi *et al* argued that pathogens readily isolated from faecal samples must be prevalent or endemic in that locality.¹⁵

Microscopy and gram staining

The microscopic characterization was carried out on the positive 129 *Salmonella* species as obtained from the previous experiment. This characterization was carried out using the Gram staining method. The isolated *Salmonella* spp were seen to be rod-shaped and most of them were scattered when critically viewed under the microscope. This is consistent with the reports by Nwabor *et al* and Akeila *et al.*, who observed that *Salmonella* spp are Gram-negative and rod-shaped bacteria that belong to the Enterobacteriaceae family.^{2,28}

Biochemical confirmation test for typhoidal *Salmonella* serovars

The biochemical confirmation test results shows that out of 129 *Salmonella* strains isolated and subjected to biochemical analysis, a

total of 81 (62 %) isolates passed the biochemical tests to confirm the presence of these *Salmonella* strains. However, 62 (48 %) isolates passed the citrate utilization test which is the only biochemical test that can phenotypically distinguish typhoidal *Salmonella* serovars (*Salmonella typhi* and *Salmonella paratyphi*) from other *Salmonella* spp.³² The result also shows that typhoidal *Salmonella* spp is glucose-positive, lactose-negative, mannose-positive, indole-negative, catalase-positive and citrate utilization-negative. This confirms the observations of previous studies.^{2, 28}

Susceptibility studies

Susceptibility studies were carried out on 62 isolates that passed the biochemical confirmation test. According to clinical laboratory standards (CLSI) *Salmonella typhi* is said to be sensitive, intermediate and resistant to ciprofloxacin and ofloxacin when the inhibition zone diameters (IZD) are ≥ 30 mm, 21-30 mm, and ≥ 20 mm respectively.²⁹ A total of 62 sample isolates were analysed using ciprofloxacin and ofloxacin disc. While 22 (35 %) were sensitive to ciprofloxacin disc, 4 (7 %) were sensitive to ofloxacin disc. Whereas 27 (44 %) showed intermediate sensitivity to ciprofloxacin, 28 (45 %) showed intermediate sensitivity to ofloxacin. Meanwhile, 13 (21 %) isolates were resistant to ciprofloxacin disc, while 30 (48 %) were resistant to ofloxacin disc. Table 1 summarises the sensitivity studies of fluoroquinolones (ciprofloxacin and ofloxacin) to typhoidal *Salmonella*. This result shows that the isolates were more resistant to ofloxacin than ciprofloxacin. While ofloxacin had the highest resistance in the range 9-20 mm with a total no of 30 isolates, ciprofloxacin had a lower resistance in the range 14 - 20 mm with 13 isolates. Table 2 shows the inhibition zones of the 10 most highly resistant isolates tested with ciprofloxacin and ofloxacin discs. The experiment was done in duplicate and the IZD was measured in mm represented in mean \pm SD.

Figure 1 presented the descriptive analysis of the zones of inhibition in percentages for isolates tested with ciprofloxacin and ofloxacin respectively. Figure 2 presents a bar chart showing the inhibition zone diameters of the 10 most highly resistant isolates to ciprofloxacin and ofloxacin. While E123 had the least inhibition zone diameter of 9.00 ± 2.83 mm, E22 had the highest inhibition zone diameter of 16.00 ± 4.24 mm. While E123 had the least inhibition zone diameter of 9.00 ± 2.83 mm, E2 had the highest inhibition zone diameter of 16.00 ± 4.24 mm. From the sensitivity studies carried out, it was observed that 35 % and 7 % of *Salmonella* isolates were sensitive, 44 % and 45 %, were intermediate, 21 % and 48 % were resistant to ciprofloxacin and

ofloxacin respectively. Although ciprofloxacin and ofloxacin belong to the same class of fluoroquinolone, ofloxacin is a later drug to ciprofloxacin and thus is expected to possess improved activity against sensitive organisms. However, this study shows that ciprofloxacin is more effective than ofloxacin for the treatment of typhoidal *Salmonella* infections.

Table 1: The summary of the sensitivity studies

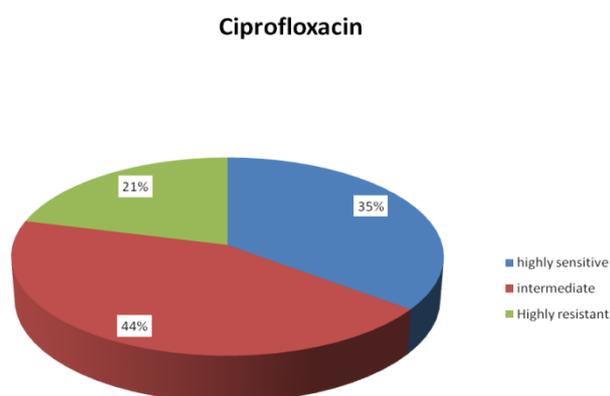
Antibiotic Disc	Antibiotic Susceptibility		
	Highly sensitive IZD ≥ 30 mm	Intermediate IZD $\leq 21-30$ mm	Highly resistant IZD ≤ 20 mm
Ciprofloxacin	22	27	13
Ofloxacin	4	28	30

Legend: Clinical and Laboratory Standards Institute (2018)

Table 2: The zones of inhibition of the 10 most highly resistant isolates tested with ciprofloxacin and ofloxacin discs. The experiment was done in duplicate and the IZD was measured in mm represented in mean \pm SD

Sample code	Antibiotic discs	Inhibition zone diameter(mm)
E22	Ciprofloxacin	16 ± 4.24
E23	Ofloxacin	10 ± 2.83
E50	Ofloxacin	11 ± 3.54
E61	Ofloxacin	13 ± 4.24
E75	Ciprofloxacin	15 ± 1.41
E92	Ofloxacin	15 ± 1.41
E98	Ofloxacin	14 ± 2.83
E111	Ofloxacin	13 ± 2.83
E118	Ciprofloxacin	14 ± 2.83
E123	Ofloxacin	9 ± 2.83

A



B

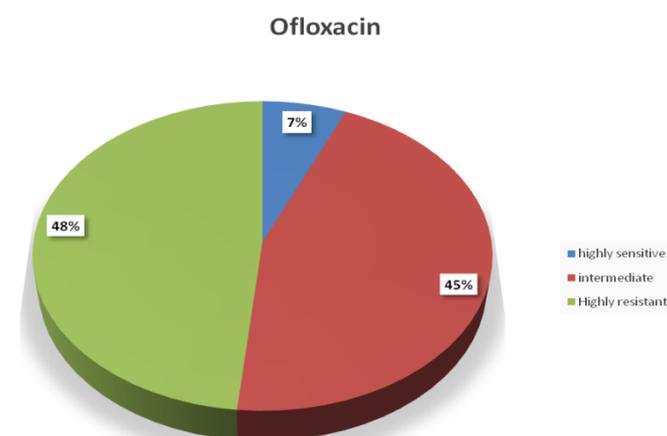


Figure 1: A pie chart showing zones of inhibition in percentages for isolates tested with A. ciprofloxacin, B. ofloxacin

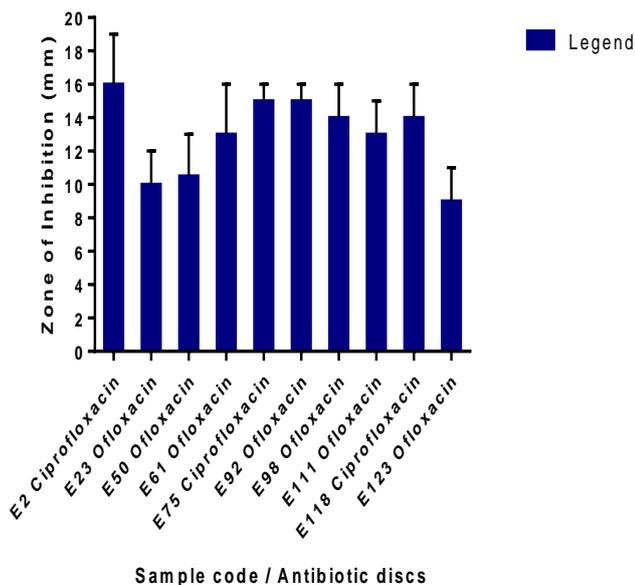


Figure 2: Inhibition zone diameters in mm of the 10 most highly resistant isolates to ciprofloxacin and ofloxacin (E1 – E300 represents the sample codes for the 300 faecal sample isolates)

Plasmid profile analysis

Figure 3 shows the plasmid bands of the standard and samples of the 10 most highly resistant *Salmonella spp* isolates as read from the ultraviolet visible spectrophotometer.

Plasmids were seen in all the 10 isolates. Each plasmid was characterized by the presence of two large plasmids bands of different sizes and migration rates. The molecular weights of the plasmids of the isolates were in the range of 31-37 kb for band 1 and 10-20 kb for band 2, this coheres to the findings of a similar study on prevalence of *Salmonella typhi* among food handlers in Nigeria.⁴ The molecular weights of the plasmid DNA band of the sample isolates were higher than that of the standard. This reveals that the test isolates contain more resistant genes than the standard. The molecular weights of the plasmids in this study were similar to the finding of previous study.¹⁵ While the highest and lowest mobility rates were 95 mm and 7 mm respectively, the highest and lowest molecular weights were 20 kb and 0.5 kb respectively. Observably, the lower the mobility rates, the higher the molecular weight of the plasmid bands. Hence, the mobility rate is inversely proportional to the molecular weight. The heavy bands as seen in E23, E50, E118, and E123 could be that the volume added, while loading in the wells exceeded the actual volume or spilled when loading in the well due to human error or that the isolates contain more resistant genes than the others. Specifically, the sensitivity studies show that E23, E50, E118 and E123 have very low IZD values of 10, 11, 14 and 9 mm respectively. Hence, this could mean that the heavier the plasmid bands the lower the IZD values and the more resistant the organisms is to the antibiotics. This means that the above isolates could contain more resistant genes, which could lead to treatment failure when these organisms are involved in salmonellosis. In plasmid band 1, while E50 and E22 had the highest mobility rate of 7mm, E92 had the least mobility rate of 3mm. Again, in plasmid band 2, whereas E123 had the least mobility rate of 16mm, E92 had the highest mobility rate of 33mm. The presence of plasmids DNA in all the resistant isolates may be responsible for their observed resistance to ciprofloxacin and ofloxacin.²² Hence, most antibiotic resistant genes are also located on plasmids. Moreover, Nordmann and Poirel observed that plasmids largely bear transferable antibiotic resistance genes.³¹

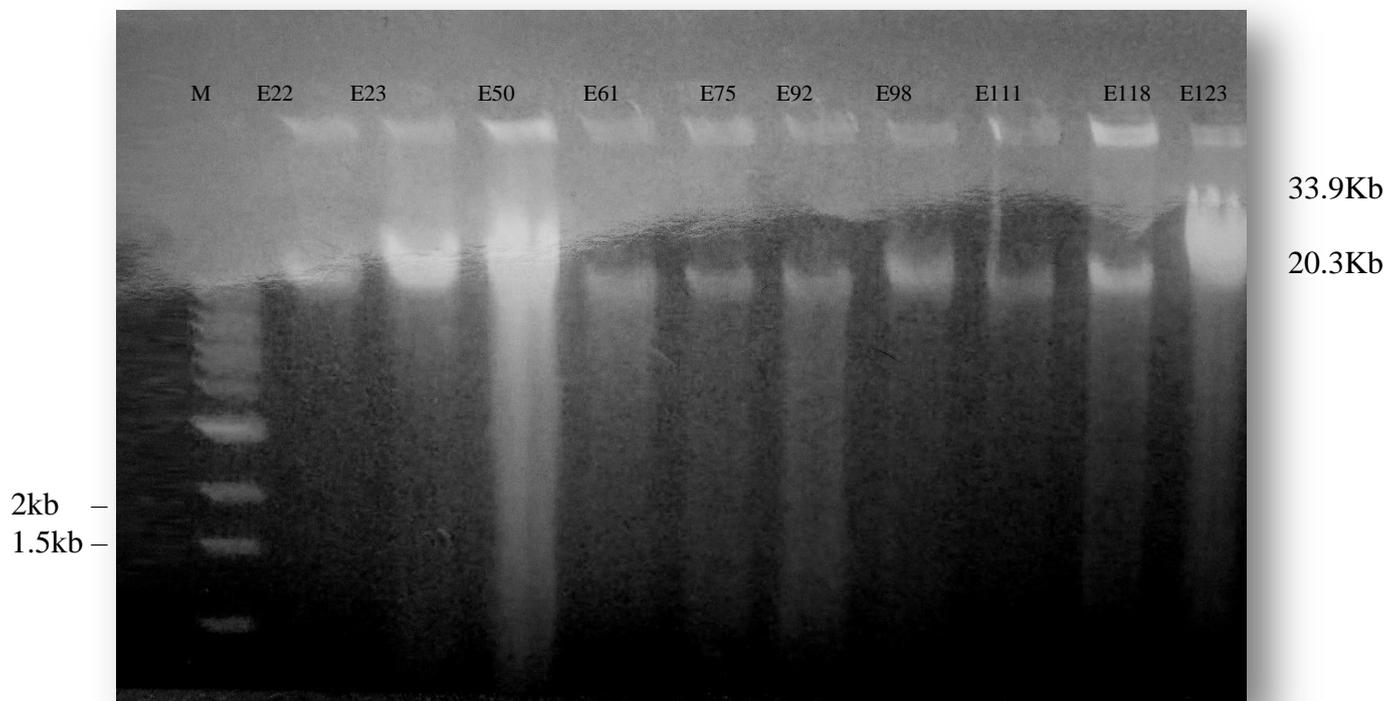


Figure 3: The plasmid bands of the standard and the samples of the 10 most highly resistant *Salmonella typhi* isolates to ciprofloxacin and ofloxacin

Plasmid curing

Table 3 shows the inhibition zone diameters obtained from the sensitivity studies repeated after curing with acridine orange on the 10 most highly resistant typhoidal *Salmonella* isolates.

All resistant isolates prior to the plasmid curing were no longer resistant as seen in Table 3. From the sensitivity studies repeated after the plasmid curing, it was observed that the IZD values improved, and the resistant isolates were either sensitive or intermediate. This shows that the plasmid DNA actually conferred resistance to the *Salmonella typhi* isolates. From the plasmid curing analysis, the susceptibility of typhoidal *Salmonella* isolates to the antibiotics (ciprofloxacin and ofloxacin) it was formerly resistant to after being cured with acridine orange shows that the genes responsible for this resistance to the selected antibiotics is plasmid mediated. Afolabi *et al.* also reported that extra chromosomal DNA (plasmids) are major mechanisms for

the spread of antibiotic resistant genes in bacterial populations.²³ The increase in resistance associated with first-line antibiotics has led to its replacement with fluoroquinolones.¹³⁻¹⁴ Ciprofloxacin and Ofloxacin are the most prescribed antibiotics for *Salmonella* infections particularly in Nigeria.²⁶

Although ciprofloxacin and ofloxacin are supposed to be prescription drugs, they are readily obtained over the counter without proper screening for the infection in Nigeria. This has invariably led to the abuse and misuse of these antibiotics by the masses. The use and misuse of antimicrobials are the major determining factors to antimicrobial resistance of bacteria in both veterinary and human medicines.^{11,23} Hence, typhoid fever remains a major public health challenge in Nigeria.¹⁷ observably typhoid fever and malaria are estimated to constitute about 50 % of all hospitalisations in Nigeria.¹⁰

Table 3: Summary of the plasmid DNA curing with acridine orange

Sample Code	Specific antibiotic Disc used	Inhibition zone	Inhibition zone	Before exposure to acridine orange	After Exposure to acridine orange
		diameter (mm) Before curing	diameter (mm) After curing		
E22	Ciprofloxacin	16	38	R	S
E23	Ofloxacin	10	25	R	I
E50	Ofloxacin	11	28	R	I
E61	Ofloxacin	13	31	R	S
E75	Ciprofloxacin	15	36	R	S
E92	Ofloxacin	15	34	R	S
E98	Ofloxacin	14	32	R	S
E111	Ofloxacin	13	30	R	S
E118	Ciprofloxacin	14	30	R	S
E123	Ofloxacin	9	22	R	I

(CLSI: ≥ 30 mm = sensitive: 21 – 30 = Intermediate: ≤ 20 mm = Resistant) S = Sensitive, R = Resistant, I = Intermediate

Conclusion

This study shows that the incidence of most commonly used fluoroquinolone resistance to typhoidal *Salmonella spp* is high in South-East Nigeria and this could be as a result of plasmid resistant DNA. This paper suggests that the presence of plasmids should be taken into consideration in antibiotic drug manufacturing in order to combat multiple drug resistance and that ciprofloxacin is more effective than ofloxacin in treating typhoidal *Salmonella* infections. Again, health agencies should intensify efforts to check indiscriminate use of ciprofloxacin and ofloxacin which increases typhoidal *Salmonella* resistance especially in low- and middle-income countries.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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