Original Research Article

Isolation of Fluoroquinolones Resistant *Salmonella* spp from Stool Samples of Patients Attending General Hospital Minna and Ibrahim Badamasi Babangida Specialist Hospital, Minna Nigeria

ABSTRACT

Typhoid fever continues to be a major health problem despite the use of antibiotics and the development of newer antibacterial drugs. This study aim was to isolate fluoroquinolones resistant *Salmonella* spp from stool samples of Patients attending General Hospital and Ibrahim Badamasi Babangida Specialist Hospital both in Minna Niger State. 450 stool samples were collected from the Hospitals. The results showed that 69 (48.00%) of the sample collected were positive for *Salmonella* species. On the basis of age children within the age range of 0-10 recorded the highest prevalence of 22.7% followed by age range 51-60 had the prevalence of 19.4%, age range >60 had the prevalence of 16.7% and age range 21-30 and 11-20 had a similar prevalence of (10.1% and 10.3%) while age range 31-40 had the least prevalence of 7.8%. There were 69 isolates of *Salmonella* species Identified, 65(94.2%) were Resistance to the antibiotics used. The highest resistance was shown to Pefloxacin 62 (89.9%) and the lowest was shown to Ciprofloxacin 27 (39.1%). *Salmonella* species exhibited 52 antibiotic resistant patterns for the ten antibiotics tested with multiple antibiotic resistance index (MARI) ranging from 0.3-1.0. Molecular analysis was carried out on 5 representative isolates to identify their strains. Polymerase chain reaction (PCR) assay showed the identified *Salmonella* strains were *Salmonella enterica* subsp. *arizonae* strain ATCC 13314, *Salmonella enterica* subsp. *enterica* serovar Typhi strain 2018K-0756, *Salmonella bongori* strain SL18, *Salmonella bongori* strain GH3Rp and *Salmonella enterica* subsp. *arizonae* strain ATCC 13314 showed resistance to fluoroquinolones.

Key: *Salmonella*, antibiotics, fluoroquinolones, resistance, prevalence

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1. INTRODUCTION

Fluoroquinolones are a class of broad-spectrum, systemic antibacterial that have long been used to treat respiratory and urinary tract infections. Fluoroquinolones are effective against both aerobic and anaerobic gram positive and gram negative bacteria, most especially *Salmonella* species. The fluoroquinolone anti-microbials incorporate ciprofloxacin, ofloxacin, perfloxacin, spartfloxacin antibiotic medications, gemifloxacin, levofloxacin and Moxifloxacin [23, 11]. They act by inhibiting the bacterial enzyme DNA gyrase, which is accountable for bacterial DNA division, winding, and alternative path during multiplication [8].

Resistance comes as a curse with antibiotics that occurs when bacteria change in some way that reduces or eliminates the effectiveness of drugs, chemicals or other agents designed to cure or prevent infections [18].

Salmonella infections in human and animal have been perceived as a major public health Issue [29, 11]. *Salmonella,* an essential occupant of the gastrointestinal tract, is perceived as quite possibly the most well-known cause of food borne diseases worldwide, bringing about millions of diseases and huge human death yearly [29, 19]. *Salmonella* species are liable for 93.8 million instances of typhoid fever and food borne infections in people and about 222,000 deaths yearly. *Salmonella* strains that are multidrug resistant (MDR) are common causes of endemic and epidemic typhoid fever infections in the community [21, 29]. There have been reports of high predominance *Salmonella* with antimicrobial resistance isolate in Nigeria [3, 6,25]. Multi Drug Resistant strain *Salmonella* is of concern not only because of its resistance to accessible fluoroquinolones, but also because it presents a risk of disease

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outbreaks that may be difficult to stop [4, 7, 6]. Such an outbreak will certainly be annihilating, particularly in developing countries where health facilities are often insufficient. This study aim to isolate fluoroquinolones resistant *Salmonella* species from stool samples of patients attending General Hospital, Minna and Ibrahim Badamasi Babangida Specialist Hospital, Minna Nigeria.

2. Materials and Methods

2.1. Study area

The study was conducted from June to December, 2019 in Minna, Niger State, Nigeria. It is situated on Latitude 9.61 N and Longitude 6.56 E at an elevation of 299 m above sea level. It is bordered to the North by Sokoto State, West by Kebbi State, and South by Kogi and South-West by Kwara State. Niger State has a common boundary with the Republic of Benin along New Bussa, Agwara and Wushishi Local Government Area. Samples were collected from selected hospitals in Minna, which included General hospital (GH) and Ibrahim Babangida Specialist hospital (IBBSH) as shown in Figure 1.

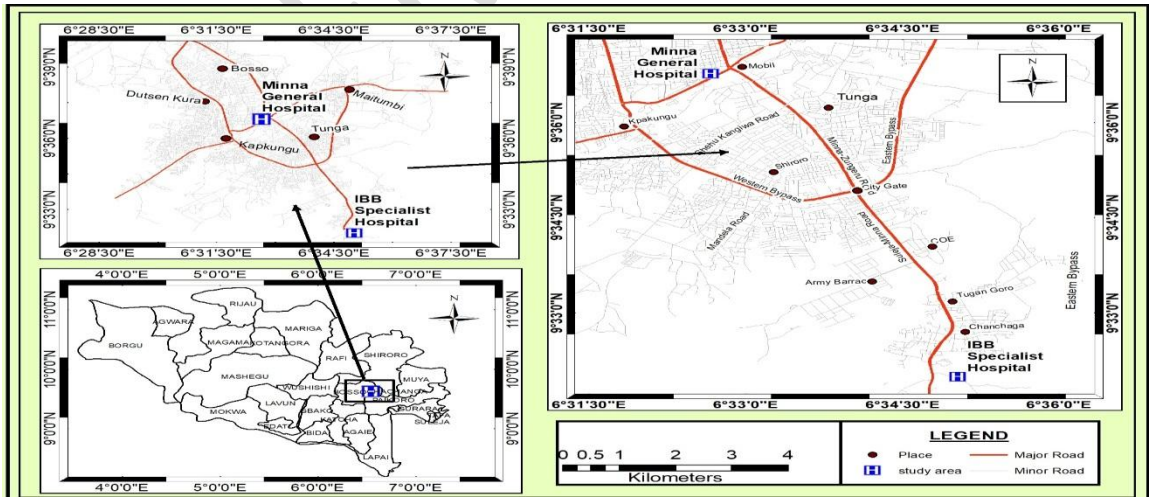

Figure 1: Study area and distribution of hospitals included in the study GH= General Hospital, IBBSH= IBB Specialist Hospital.

2.2.

2.3. Sample collection

Four hundred and fifty (450) stool samples were collected from patients attending selected hospitals in Minna. Ethical clearance was obtained from the research and ethics committee of the Niger State Hospitals Management Board for the study. Two hundred and fifty (250) stool samples from GH, and two hundred (200) from IBBSH. The stool sample collected on a sterile container was kept at 4^{o}C in a Coleman box and transported to the Laboratory for processing. Each of the samples collected was appropriately labeled. All the samples were taken to Microbiology Laboratory Federal University of Technology Minna Niger Sate and processed according to standard microbiological procedure [9].

2.4. Isolation and Identification of *Salmonella* sp.

Salmonella species were isolated according to the methods outlined by [9]. An aliquot of stool sample was inoculated into 9ml of Selenite F Broth and incubated at 37^{o}C for 24 hours. A loopful from the above was streaked onto *Salmonella Shigella* Agar (SSA) and the plates incubated overnight at 37^{o}C for 24 hours. Colonies (one per plate) that showed characteristic black doted center with or without transparent borders on SSA were subculture on *Salmonella Shigella* Agar (SSA) and the plates incubated overnight at 37^{o}C for 24 hours. And subsequently sub cultured into nutrient agar slants and was kept at 4^{o}C for further analysis. The pure isolates obtained were identified and characterized based on their cultural (colony) characteristics, Gram stain and biochemical properties [9].

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2.5. Antibiotic Susceptibility Test

Kirby Bauer's disc diffusion technique was used for antimicrobial susceptibility testing. The results were interpreted using [10]. Briefly, individual colonies were suspended in normal saline to 0.5 McFarland standards. Using a sterile swab, the suspensions was inoculated on Muller Hinton agar and incubated at 37 ˚C for 24 hours. Commercially available antibiotic Chloramphenicol (CH) 30 µg, Septrim (SXT) 30 µg, Ciprofloxacin (CPX) 10 µg, Gentamicin (CN) 10 µg, Amoxacillin 30µg, Augmentin 30µg, Ofloxacin (OFX) 10 µg, Streptomycin 30 µg, Spartfloxacin 10 µg, Pefloxacin 30 µg discs of the drugs commonly used for treatment of Gram-negative bacterial infections were tested. The zone of inhibition was measured and used to classify the organisms as susceptible, intermediate or resistant to a specific antibiotic according to [10].

Multiple Antibiotic Resistance Index (MARI) in respect to a single isolate defined as a/b, where “a” represents the number of antibiotics to which the isolate was resistant and “b” represents the number of antibiotics to which the isolate was exposed, was also calculated [22].

2.5. Molecular identification of Fluoroquinolones resistance *Salmonella* strain

In order to isolate the Fluoroquinolones Resistance *Salmonella* species, the following steps was performed as described below.

2.5.1. Bacteria cell preparation

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The preparation of the cell was carried out using the method described by [13]. Lauria Bertani (LB) broth Medium was prepared comprising of 1.5g peptone water, 0.5g yeast extract, 0.5g sodium chloride, 1ml of 1N sodium hydroxide, was dissolved in distilled water and with a volume of up to 100ml. Five milliliter (5ml) of the LB medium was dispensed into each universal sample bottle and autoclaved at 121^{o}C for 15 minutes to sterilize. A single colony was picked from freshly streaked isolate on *Salmonella Shigella* Agar (SSA) plate and inoculated into the universal sample bottles and incubated at 37^{o}C for 24 hours.

2.5.2. DNA extraction

The [28] protocol was used to extract DNA. Single medium-grown colonies after 48 hours at 28 degrees Celsius, the plants were shaker-grown after being transferred to 1.5 mL liquid medium. For a total of 5 minutes, the cultures were centrifuged at 4600rpm. At a temperature of 520 l, to resuspended the pellets, TE buffer was used (10 mMTris-HCl, 1mM EDTA, pH 8.0). After that, 3 liters of Proteinase K (20 mg/ml) and 15 microliters of 20% SDS were added. 100 l of 5 M NaCl and 80 l of 10% CTAB solution at 0.7 M NaCl were used to vox the mixture after 1 hour of incubation at 37 ^{o}C.

After being incubated at 65°C for 10 minutes, the suspension was kept on ice for 15 minutes. After 5 minutes on ice, the cells were centrifuged at 7200g for 20 minutes. Isoamyl alcohol (24:1) was applied in a similar amount to the chloroform. After that, the aqueous phase was moved to a new tube, where isopropanol (1: 0.6) was applied and DNA was precipitated at 20 ° C for 16 hours. DNA was removed using a 13000g centrifuge for 10 minutes, followed by

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washing with 500 liters of 70% ethanol, air drying for three hours at room temperature, and 50 liters of TE buffer dissolved in water.

2.5.3. Polymerase chain reaction

A total of 10 liters of 5 x GoTaq colorless reactions, 3 liters of 25 mM MgCl2, 1 liter of 10 mM dNTP mix, and 1 liter of 10 pmol per 27F primer made up the PCR sequencing preparation cocktail. Taq DNA polymerase (Promega, USA) primers -1525R, 5'-AAGGGTGATCCAGCC-3' and 5'-AGA GTT TGA TCM TGG CTC AG-3' were used to make 42 liters of sterile distilled water (Wawrik *et al.,* 2005). The PCR was performed on an Applied Biosystems Inc. GeneAmp 9700 PCR Machine Thermalcycler with a PCR profile that included a 5-minute 94°C denaturation for 30 seconds, 30 periods at 94°C for 30 seconds, followed by 30 intervals at 94°C for 30 seconds, 50°C for 60 seconds, and 72°C for 1 minute 30 seconds, with a 10-minute final termination at 72°C. At a chilling 4 degrees Celsius, no less. GEL, unwind [15].

2.5.4. Integrity test

A 1 percent Agarose gel was used to check the integrity of the amplified 1.5Mb gene fragment to validate amplification. The buffer was used to produce a 1.5 percent agarose gel (1XTAE buffer). In an oven, for 5 minutes, the suspension was boiled. Until staining with 0.5 g/ml ethidium bromide 3l, before being used, the molten agarose had to cool to 60°C (which absorbs UV light that is invisible to the naked eye and transmits the energy as visible light visible to the naked eye). Molten agarose was poured into the casting tray using a comb inserted into the holes. To create the wells, the gel was allowed to solidify for 20 minutes. The gel was barely

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dissolved in the 1XTAE buffer poured into the gel tank. After well loading of the 100bp DNA ladder 1, two microliters of 10X blue gel filling dye (2 l) were added to 4l of PCR products, which provided color and density to make loading and controlling the gel easier. It was a gel electrophoresed and photographed for 45 minutes at 120V, with data shown using UV trans-illumination. The sizes of the PCR products were determined using the mobility of a 100bp molecular weight ladder in the gel, which ran alongside experimental samples.

2.5.5 Purification of the amplified Product

The pieces that had been amplified were distilled to clear the PCR reagents, ethanol was used after gel integrity. Every approximately 40 l PCR amplified product was inserted in a new 1.5 l sterile eppendorf tube with 7.6 l of 3M Na acetate and 240 l of 95 percent ethanol, thoroughly vortexed, and stored at -20 °C for at least 30 minutes. Centrifuge the pellet at 13000 g and 4 ° C for 10 minutes, then discard the superfluous (invert the tube into the garbage), wash the pellet with 150 l of 70% ethanol, 15 minutes at a weight of 7500 g and a temperature of 4 ° C Until sequencing, remove all supernatant (invert tube on trash) and dry tube for 10-15 minutes in a fume hood at room temperature, store at -20°C in 20 liters of sterile distilled water. A nano drop from the thermo-scientific model 2000 was used to quantify the filtered fragment and tested on a 1.5 percent Agarose gel for 1 hour at 110V to confirm the existence of the distilled content.

2.5.6. Sequencing

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The amplified fragments were sequenced using an Applied Biosystems Genetic Analyzer 3130xl sequencer and the Large Dye terminator v3.1 cycle sequencing kit, in accordance with the manufacturer's instructions. All genetic studies were carried out using Bio-Edit software and MEGA 6. DNA is quantitated, fragmented and tagged with adapter sequences added to the ends (Nextera XT Library Prep Kit, Illumina, Inc.). The library is amplified by PCR followed by cleanup and size selection using AMPure XPTM (Beckman Coulter, Inc.) beads to remove very short library fragments from the sample. Library normalization is performed using the Nextera kit to ensure equal library representation in the pooled sample. The pooled amplicon library (PAL) is performed by combining equal volumes of normalized library, quantitated, and diluted in hybridization buffer forming a diluted amplicon library (DAL). This DAL is heat denatured and loaded onto the MiSeq reagent cartridge for sequencing [5].

2.6. Statistical Analysis

Isolates were classified as resistant, intermediate and sensitive using CLSI 2019 guide for the interpretation of zones of inhibition

3. Results

Out of 450 samples collected, the prevalence of *Salmonella* species in selected hospitals was 30.4%. As identified in Table 1. General Hospital Minna Niger State accounted for the high number of isolates 16.4% (41/250) while IBB Specialist Hospital Minna Niger State had the low number of isolates 14.0% (28/200). The age-related prevalence rate of *S.* species was determined (Table 4.2). The age group 0-10 years has the highest prevalence rate (22.7%), followed by the age group 51-60 years (19.4%), and the age group >60 years has a

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prevalence rate of (16.7 percent), age group 11-20 years has prevalence of (10.3%), age group 21-30 years has prevalence rate of 10.1% (7/69) while the least prevalence was age group 31-40 years 7.8 (5/64) as shown in Table 2. Prevalence of *Salmonella* on the basis of gender is presented in Table .3. The females had a higher prevalence rate of (15.7%) while males had lower prevalence rate of (14.9%). The results of antimicrobial susceptibility tests show that Streptomycin and Ofloxacin has the highest susceptibility 49.3% and 44.9% follow by Sparfloxacin 33.3%, Ciprofloxacin 31.9%, Septrim 31.9%, Chloramphenicol 30.4%, Gentamycin 30.4%, Amoxicillin 29.0% while the least susceptibility (high resistant) against Pefloxacin 10.1% and Augmentin 14.5%.

The result also showed that Pefloxacin and Augmentin had the highest resistant of 89.9% and 73.9%, follow by Sparfloxacin 60.9%, Septrim 58.0%, Chloramphenicol 56.5%, Gentamycin 56.5%, Amoxicillin 52.2%, Streptomycin 47.8%, Ofloxacin 43.5% while the least resistant was Ciprofloxacin 39.1% as shown in Table 4 and Table 5.

figure 2 Shows the Agarose gel electrophoresis indicating a positive amplification of the 16s rRNA regions of *Salmonella* species isolate. The gel shows white horizontal lines on lane Mk 1, 2, 3, 4 and 5. This indicates the presence of the strain in all isolates as shown in figure 2.

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Table 1. Prevalence of *Salmonella* species among the selected hospitals

Sample sources	Total sample screened (%)	Number Positive	Prevalence (%)

Gen. Hospital	250 (55.56)	41	16.4
IBB	200 (44.44)	28	14.0
Total	450 (100)	69	30.4

Table 2. Prevalence of *Salmonell*a species based on age group

Age (Years)	**Number**	**Number of isolated *Salmonella***	**Prevalence (%)**
0-10	157	35	22.3
11-20	68	7	10.3
21-30	69	7	10.1
31-40	64	5	7.8
41-50	44	6	13.6
51-60	36	7	19.4
>60	12	2	16.7

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Table 3. Prevalence of *Salmonell*a species based on gender

Gender	**Number of samples**	**Number of Isolated *Salmonella***	**Prevalence (%)**
MALE	202	30	14.9

FEMALE	248	39	15.7

Table 4. Antibiotics Susceptibility of *Salmonella* isolates

ANTIBIOTICS	S	I	R
CPX (%)	22(31.90)	20 (29.00)	27(39.10)
PEF (%)	7 (10.10)	0 (0.00)	62(89.90)
OFX (%)	31(44.90)	8(11.60)	30(43.50)
SP (%)	23(33.30)	4(5.80)	42(60.90)
CN (%)	21(30.40)	9(13.00)	39(56.50)
S (%)	34(49.30)	2(2.90)	33(47.80)
AM (%)	20(29.00)	13(18.80)	36(52.20)
SXT (%)	22(31.90)	7(10.10)	40(58.00)
CH (%)	21(30.40)	9(13.00)	39(56.50)
AU (%)	10(14.50)	8(11.60)	51(73.90)

Table 5. Antibiotics mean diameter zone of inhibition

Antibiotics	Disk content	mean diameter zone of inhibition (mm)	Sensitivity pattern
Ciprofloxacin	5ug	17.3±1.1	I
Peflacin	5ug	14.1±1.0	I
Ofloxacin	5ug	14.1±1.1	R
Spafloxacin	5ug	12.2±1.0	R
Gentamycin	10ug	11.4±0.9	R
Streptomycin	10ug	12.6±1.0	I
Amoxil	20/10ug	12.3±1.2	R
Septrin	1.25/23.75ug	10.8±1.0	I

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Chloramphenicol	30ug	11.2±0.9	R
Augumentin	20/10ug	7.6±0.9	R

KEY: R= Resistant, I= Intermediate

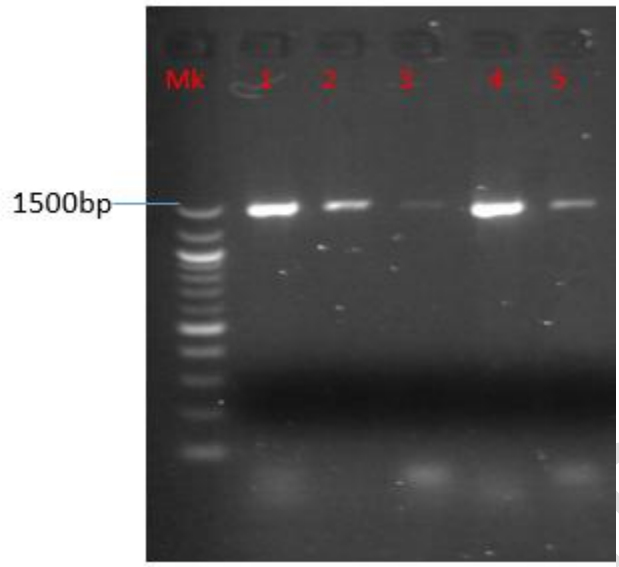

Figure 2. Agarose gel electrophoresis indicating a positive amplification of the 16s rRNA regions of bacteria isolate.

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4. DISCUSSION

In this study, four hundred and fifty stool samples (450) were obtained from patients attending General Hospital Minna and IBB Specialist Hospital Niger State. The prevalence of *salmonellosis* was 30.4%. This was relatively low when compare with a prevalence of 45.0% reported by [3] in Minna Nigeria while [16] had a higher prevalence of 66.7% in Imo Nigerian. The differences in the prevalence rate could be due to differences in sample size and technique for isolation. This is in agreement with [12] who reported that the differences in the prevalence assessment could be due to differences in management system, sample size, and method of isolation.

The prevalence of Typhoid fever recorded in General Hospital was 16.4% while IBB Specialist Hospital was 14.0%. It shows that General Hospital had higher prevalence of Typhoid fever while IBB Specialist Hospital had lower prevalence. This could be due to the fact that General Hospital had higher number of sample collection than IBB specialist. This could likewise be due to patronage; most patients prefer general Hospital than IBB Specialist due to the high cost of transportation.

In this study, children within the age range of 0-10 recorded the highest prevalence of 22.7% followed by age range 51-60 had the prevalence of 19.4%, age range >60 had the prevalence of 16.7% and age range 21-30 and 11-20 had a comparative prevalence of (10.1% and 10.3%) while age range 31-40 had least prevalence of 7.8%. The high prevalence of children aged 0 to 10 could be attributed to the fact that children are the most vulnerable group in an area where there is lack of clean water and poor environmental hygiene. It could also be due to the fact that children aged 0 to 10 immune systems are not fully developed and they do not observe personal hygiene. This research agreed with [29] findings that indicate a high prevalence of Typhoid fever in children aged 5 to 10.

This study shows a higher prevalence rate among females than males. Females recorded prevalence rate of 15.7% while males recorded 14.9%. This is consistent with studies by [20] in Imo State, [14] in Abia State, and [2] in Karu, Nasarawa State Nigeria. Notwithstanding, different studies led by [24] in Abeokuta, Ogun State and [17] in Biu, Borno State reported a higher prevalence rate in males. This difference recorded in the gender related prevalence of Typhoid fever in this study could be ascribed to Immune system. This is in agreement with the finding by [30] who reported that different records

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including social practices, environment, and immunological abilities could influence gender statuses with disease conditions.

In this study, *Salmonella* species showed a high degree of antibiotic resistance to commonly used antibiotics. This improvement as noticed might be recognizable to wrong and inaccurate diagnosis and abusive utilization of the accessible antibiotics bringing about the turn of events and spread of multidrug resistant strains of *Salmonella* isolate. This is in line with the previous WHO study [29], which state that the misuse of available antibiotics caused events to shift and the spread of *Salmonella* isolate multidrug resistance strains.

The results of antimicrobial susceptibility tests show that Streptomycin and Ofloxacin had the highest susceptibility 49.3% and 44.9% follow by Sparfloxacin 33.3%, Ciprofloxacin 31.9%, Septrim 31.9%, Chloramphenicol 30.4%, Gentamycin 30.4%, Amoxicillin 29.0% while the least susceptibility (high resistant) was recorded against Pefloxacin 10.1% and Augmentin 14.5%. The result likewise showed that Pefloxacin and Augmentin has the highest resistant of 89.9% and 73.9%, follow by Sparfloxacin 60.9%, Septrin 58.0%, Chloramphenicol 56.5%, Gentamycin 56.5%, Amoxicillin 52.2%, Streptomycin 47.8%, Ofloxacin 43.5% while the least resistant was against Ciprofloxacin 39.1%.

In this study, the Fluoroquinolones showed a higher resistant to pefloxacin and Sparfloxacin. This high resistance of *Salmonella* isolates to antibiotics could be because of abuse and misuse of drugs. It could likewise be because of the accessibility of the drugs to patients due to its more affordable and regularly sold without prescription. Comparable study was accounted for by [26] who expressed that resistance is associated with the

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accessibility of these drugs to individuals since it is more affordable in the neighborhood pharmacies and regularly sold without prescription.

The result of this study shows that ofloxacin and ciprofloxacin had activity against Typhoid fever, in contrast to high resistance to ciprofloxacin reported by [26] in Nassarawa State, Nigeria. This might be due to the way the antibiotic resistant genes have modified the microbial community by consistent antibiotic use and the impacts actually continue for quite a long time even after stopped use. If treatment with an antibiotic is stopped for a long period of time, an organism that was previously resistant to it can become susceptible. Similarly, [27] state that organisms that were previously resistant to a specific antimicrobial may become susceptible if treatment with the antibiotic is discontinued for an extended period of time.

Polymerase Chain Reaction detection of fluoroquinolones resistance strains investigation revealed in figure 2. *Salmonella enterica* subsp. *arizonae* strain ATCC 13314, *Salmonella enterica* subsp. *enterica serovar Typhi* strain 2018K-0756, *Salmonella bongori* strain SL18, *Salmonella bongori* strain GH3Rp and *Salmonella enterica* subsp. *arizonae* strain ATCC 13314. The presence of these resistance strains of *Salmonella* could be because of the presence of the genes on plasmid carrying various resistance genes. According to [1] the presence of the genes is a significant recognizable proof marker in distinguishing serious *Salmonella* infection.

5. CONCLUSION

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Prevalence rate of *Salmonella* isolates in this study was (30.4 %). *Salmonella* isolate was observed to be resistant to fluoroquinolones antibiotics. This study revealed that *Salmonella enterica* subsp arizonae, *Salmonella enterica* enterica serovar Typhi, *Salmonella bongori* was present by Molecular identification. The *Salmonella* species possess resistance to fluoroquinolones and other antibiotics. The presence of these resistance strains confirms that the isolates were fluoroquinolones resistant.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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