IJMPR 2021, 5(2), 10-16

International Journal of Modern Pharmaceutical Research
www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article

SJIF Impact Factor: 5.273

ANTIBIOTIC SUSCEPTIBILITY AND MOLECULAR DETECTION OF FLUOROQUINOLONES RESISTANCE GENES FROM *SALMONELLA TYPHIMURIUM* ISOLATED FROM POULTRY FARMS IN KEFFI, NIGERIA

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Received on: 27/01/2021
Revised on: 17/02/2021
Accepted on: 07/03/2021

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ABSTRACT

Antibiotic susceptibility and molecular detection of fluoroquinolones resistance genes from *Salmonella typhimurium* isolated from Poultry farms in Keffi, Nigeria. A total of one hundred and sixty (160) samples of feacal droppings were collected from different poultry farms and *Salmonella typhimurium* were isolated and identified using standard microbiological methods. Fluoroquinolone resistant genes (*parE, gyrB* and *gyrA*) were carried out using polymerase chain reaction (PCR) method. The occurrence of *Salmonella typhimurium* was 24.4%. The occurrence of *Salmonella typhimurium* in respect to different poultry farm was higher in farm C (32.5%) and lower in farm D with (15%). In this study Antibiotics Resistance shows that *Salmonella* isolates were resistant to Ciprofloxacin, Cefotaxime and Ofloxacin. Antibiotics resistant phenotype against *Salmonella* isolate were distributed into different antibiotic resistance pattern and the most common pattern were STX-CTX-NOR-AMC-CXM-CIP-C with 12.8% Resistance and CTX-NOR-AMC-CXM-TET-CIP-C with 1.03% Resistance. The multiple Antibiotic Resistance (MAR) Index of *Salmonella* species isolated was common at ≥0.7 with 38.5%. fluoroquinolone resistant genes *gyrA, gyrB*, and *parE* were detection from isolates that were highly resistant to quinolone drugs. Poultry farmers and handlers should ensure proper hygiene to avoid contamination in poultry farms and poultry products.

KEYWORDS: *Salmonella,* Antibiotic Resistance, *parE, gyrB* and *gyrA,* poultry farms.

INTRODUCTION

Background of the Study

The incidence of food borne diseases in humans has increased considerably in the last few years. Poultry products have been repeatedly implicated in food borne infections.[1] Poultry can harbour different food borne pathogens. Many reports in recent years have shown that *Salmonella* and *Campylobacter* spp are the most common causes of human food borne bacterial diseases linked to poultry.[1] Poultry products are considered as some of the main carriers of *Salmonella typhimurium* and represent a significant share of the attributed sources of Salmonellosis in humans.[2] The widespread occurrence of *Salmonella* in the environment and the intensive husbandry practice used in the meat, fish and shellfish industries have been a significant problem in public health.[3]

Non-typhoidal *Salmonella* are zoonotic pathogens and are the important causes of food borne infection because *Salmonella* have a broad host range and are strongly associated with animal and plant product.[2] *Salmonella typhimurium* and *S. enteritidis* are natural pathogen of birth and beef which are common reservoirs for human infection.[4] The infections due to NTS range from mild diarrheal disease to severe systemic Infection, and are of significant public health concern globally,[5] Non-typhoidal *Salmonella* infections are transmitted to humans primarily through consumption of contaminated foods from animal origin.[6] Therefore, this study focuses on antibiotic susceptibility and molecular detection of Fluoroquinolone resistant genes of *Salmonella typhimurium* isolated from Poultry farms in Keffi, Nigeria

METHODS

Sample Collection

Feacal droppings (n=160) was collected in a sterile universal bottles the samples were transported to the laboratory immediately after collection in an insulating foam box with ice maintaining the temperature from 4°C to 6°C.

Isolation and Identification of *Salmonella typhimurium*

Isolation of *Salmonella typhimurium*

The isolation of *Salmonella typhimurium* was carried out as described by.[7] The samples were first enriched by

inoculating it into 5ml of Selenite Fluid Broth (SFB) in Bijou bottle and were incubated at 37^0 C for 24 hours. The 24 hours Selenite Fluid Broth cultures were inoculated into petri dishes containing sterile Bismute sulphite Agar using an inoculating loop. The plate was inoculated at 37^0C for 24 hours. Specimens from the Bismute sulphite medium was sub-cultured for biochemical tests to confirm the isolates as *Salmonella* Typhimurium as described by.[8]

Gram Staining and Microscopy

Air dried smear of the isolate obtained was fixed and stained with crystal violet solution (primary dye) for 60 seconds, rinsed with tap water and drained to avoid diluting with the mordant. It was further flooded with iodine solution (mordant) for 60seconds and rinsed. Acetone was applied drop wise on the tilted slide for 2-3 seconds until all free colours were removed and subsequently rinsed with tap water. The slides was further flooded with safranin for 20-30 seconds, rinsed and air dried. Finally, all slides were examined under the microscope at x100 objective lens Cheesbrough, (2006).[8]

Biochemical Screening Test of *Salmonella typhimurium*

The following biochemical tests was carried out as recommended for the biochemical screening of *Salmonella typhimurium*; TSI, Urease, Indole, Citrate, Voges Proskauer, and methyl red (IMVIC).

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing of the bacterial isolates was carried out as earlier described by the Clinical and Laboratory Standards Institute.[9] Briefly, three (3) pure colonies of the isolates were inoculated into 5 ml sterile 0.85% (w/v) NaCl (BDH chemical Ltd, England) (normal saline) and the turbidity of the bacteria suspension was adjusted to the turbidity equivalent to 0.5 McFarland's standard. The McFarland's standard was prepared as follows: 0.5 ml of 1.172% (w/v) BaCl2.2H2O (BDH chemical Ltd, England) was added into 99.5 ml of 1% (w/v) H2SO4 (BDH chemical Ltd, England.

Antimicrobial SusceptibilityTesting

The antibiotic discs (Oxoid, UK) five (5) per plates were evenly dispensed unto the surface of the inoculated Agar plate using a sterile forceps and gently pressed down to ensure complete contact with the Agar surface. The plates were inverted and incubated at 37ºC for 18 - 24 hours. *In-vitro* susceptibility of *Salmonella* isolates to the antibiotics drugs were performed by the standard modified disc diffusion of Kirby- Bauer technique using guidelines established by CLSI (2016).The antibacterial agents that were employed are: Amoxicillin- clavulanic acid(AMC) 30µg, Chloramphenicol (C) 30 µg, Sulphamethoxazole- Trimethroprim (SXT) 25 µg, Ciprofloxacin (CIP) 5 µg, Cefotaxime (CTX) 30µg, Gentamicin (CN) 10 µg, Cefuroxime (CXM), 30µg, Tetracycline (TET) 10µg, Ofloxacin (OFX) 5 µg, , Norfloxacin (OFX) 10 µg, (Oxoid, U.K).

Determination of Multiple Antibiotic Resistances in the Isolates

The MAR index was determined for all isolates resistant to at least two of the antibiotics tested as follows.[10]

$$\text{MAR Index} = \frac{\text{No. of antibiotics isolate is resistant to}}{\text{No. of antibiotics isolate is exposed to}}$$

Detection of Fluoroquinolones Resistance Genes in Isolates

Bacteria Cell preparation

The preparation of the cell was carried out using the method described by.[11] Luria Bertani (LB) broth Medium was prepared comprising of 1.5g peptone water, 0.5g yeast extract, 0.5g sodium chloride, 1ml of sodium hydroxide, was dissolved in distilled water and made up to the volume of 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 xylose deoxycholate agar plate and inoculated into the universal sample bottles and incubated at 37^{o}C for 24 hours.

Extraction of DNA

1ml of the cultured broth was dispensed to each 1.5ml Eppendorff tubes, the tube were centrifuged at 10000g for 1 minute, the supernatant was discarded, and the pellets were re-suspended in 600µl lysis buffer and incubated at 56^{o}C for 30 minutes. The mixture was transferred to a spin column in a collection tube and centrifuged at 10000g for 1minute, flow through together with the column was discarded. The spin column thereafter was inserted into a new collection tubes. Five hundred (500) µl of wash buffer 1 was added and centrifuged at 10000g for 1minute, after which flow through was discarded, and 500µl of wash buffer II was also added and centrifuged at 10000g for 1 minute, and the flow through discarded as before spin column was then centrifuged empty at 10000g for 2 minutes to remove residual alcohols and salt, the collection tube was discarded and the spin column inserted into a 1.5ml micro-centrifuge Eppendorff tube. One hundred (100) ml of elution buffer was added to the spin column and incubated at room temperature for 2 minutes and centrifuged at 10000g for 2 minutes, and then the spin column was discarded. The resultant DNA in 1.5ml micro-centrifuge tube was stored in -86^{o}C refrigerator for later use in the downstream application.

PCR Amplification

Polymerase Chain Reaction was carried out in a total volume of 25µl containing 12. 5µl of master mix containing taq (*thermosaquaticus*) polymerase, dNTPs and 2mM $MgCl_2$ was mixed with 7µl template DNA. One (1) µl forward, 1µl reverse primer and 3.5µl nuclease free water in a PCR micro tube as for the multiplex PCR (*gyrA, gyrB, parC and parE*) primer, the

PCR conditions was initial denaturation at 94 ^{0}C for 5-10 mins followed by 40 cycle of denaturation at 94^{0}C for 30 s, annealing at 58^{0}C for 30sec and extension at 72 ^{0}C for 30 sec. Final extension at 72^{0}C for 7 mins and finally the reaction was held at 4^{0}C.[12]

The cycling parameter for *par* E involved initial denaturation at 95^{o}C for 5 minutes followed by 30 cycles of denaturation at 95^{o}C 30 seconds, annealing at 58^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds. This was followed by the final extension at 72^{o}C for 7 minutes and finally there action wash old at 4^{o}C.[14] The second well was loaded with negative control then followed by PCR amplicons. PCR was performed in a DNA thermal cycler (Gene Amp PCR system 9700, applied bio system) according to the manufacturer instructions. The primers used for PCR was as contained to the value giving.

Agarose Gel Electrophoresis

The casting apparatus was assembled with the combs placed in position, 1.5g of Agarose powder was weighed and 100ml of 1× TBE buffer was added, the mixture was micro waved and cooled to 65^{o}C. Five (5) µl of ethidium bromide was added and shaken so that it was uniformly distributed. The preparation was carefully poured into the casting tray and allowed to solidify with the combs in place. The combs was carefully removed and the gel tray was removed and transported to electrophoretic tank containing 1× TBE buffer, the volume of the buffer adjusted to the maximum.

If wells are loaded with samples, control and DNA ladder, Voltage was set at 80v for 45 minutes. The gel tray was taken into gel documentation units, the gel was carefully slipped down from the tray to the gel documentation system, and the gel was viewed and documented in a computer system.

Table 1: Primers and target genes with amplicon sizes for Antibiotic Resistance Genes.

Genes	Primer sequence (5'-3')	Amplicon size	Reference
gyrA (F)	5´-CGTTGGTGACGTAATCGG-3´	972	[13]
(R)	3-CCGTACCGTCATAGTTAT-5´		
gyrB (F)	5´-GCGCTGTCCGAACTGTACCT-3´	615	[13]
(R)	3´-GGTGATCAGCGTCGCACTTCC-5´		
parC (F)	5´-ACGCTGTTGTTAGGAAGTG-3´	857	[13]
(R)	3´-TTGAGGCTGGGTGAAGT-5´		
ParE (F)	5´-TCTCTTCCGATGAAGTGCTG-3´	768	
(R)	3´ATACGGTATAGCGCGGTAG-5´		

Key: F = Forward; R = Reverse

RESULTS AND DISCUSSION

Out of 160 feacal dropping samples obtained from different farms, the occurrence of the isolates was 24.4%. The occurrence of the isolates with relation to different farms showed that the isolates from feacal droppings was high in farm C 32.5% but low in farm D with 15% as shown in Table 2. The *Salmonella* isolates were more resistant to Cefotaxime 89.7%, but less resistance to Ofloxacin 30.8%.

Antibiotic resistant phenotype against *Salmonella typhimurium* as shown in table 4 the isolates were distributed into different antibiotic resistance pattern and the most common pattern were STX-CTX-NOR-AMC-CXM-CIP-C with 12.8% Resistance and CTX-NOR-AMC-CXM-TET-CIP-C with 1.03% Resistance.

The multiple indices of the *Salmonella typhimurium* isolate is shown in the table 5. The isolates with MAR ≥ 0.2 are defined as MAR isolates and the commonest MAR index was 0.7 with 38.5%.

Molecular detection of quinolone gene in twelve (12) isolates that were highly resistant to quinolones antibiotics showed that Lanes1, 3, 4, 5, 6, 7, 8 and 12 had *pare* gene as showed in plate 1; Lanes 1, 3 4 and 12 represent *gyrB* gene as shown in plate 2 and Lanes 2, 4, 8 and 9 represent *gyrA* gene as shown in Agarose Gel Electrophoresis plate 3.

Table 2: Occurrence of *Salmonella typhimurium* from samples in relation to farms in Keffi, Nasarawa State, Nigeria.

Farms	No. of Samples Collected	No. (%) Isolates from Feacal droppings (n=39)
A	40	8 (20%)
B	40	12 (30%)
C	40	13 (32.5%)
D	40	6 (15%)
Total	160	39 (24.4%)

KEYS: A = Agwan Ninzo, B = Jigwada, C = Gauta, D = Tunayi

Table 3: Antibiotic Resistance of *Salmonella typhimurium* isolated from poultry farms in respect to feacal droppings in Keffi, Nasarawa state, Nigeria.

Antibiotic	Disc Content (µg)	Resistance in Feacal droppings
CN	30	17 (43.6)
STX	25	26 (66.7)
OFX	5	12 (30.8)
CTX	30	35 (89.7)
NOR	15	23 (59.0)
AMC	30	28 (71.8)
CXM	30	34 (87.2)
TET	30	17 (43.6)
CIP	5	32 (82.1)
C	15	23 (59.0)

Key: CN = Gentamicin; STX = Trimethroprim-sulfamethoxazole, OFX = Ofloxacin; CTX = Cefotaxime; NOR = Norfloxacin; AMC = Amoxicillin-Clavulanic acid; CXM = Cefuroxime, TE = Tetracycline, CIP = Ciprofloxacin; C = Chloramphenicol.

Table 4: Antibiotic Resistance Pattern of*Salmonella* typhimurium isolated from poultry farms in Keffi, Nasarawa State, Nigeria.

S/N	Antibiotic Resistance phenotypes	No. (%) Isolate from feacal droppings(n=39)
1	STX-CTX-NOR-CXM-CIP-C	1(2.6)
2	CTX-AMC-CXM-TET-CIP-C	2(5.1)
3	CTX-NOR-CXM-TET-CIP-C	0(0.0)
4	CTX-NOR-AMC-CXM-CIP-C	1(2.6)
5	STX-CTX-NOR-AMC-CXM-CIP	2(5.1)
6	STX-OFX-CTX-AMC-CXM-CIP-C	1(2.6)
7	STX-CTX-NOR-AMC-CXM-CIP-C	5(12.8)
8	CTX-NOR-AMC-CXM-TET-CIP-C	4(1.03)
9	STX-CTX-AMC-CXM-TET-CIP-C	2(5.1)
10	STX-OFX-CTX-AMC-CXM-TET-CIP	1(2.6)
11	CN-STX-CTX-NOR-AMC-CXM-CIP	2(5.1)
12	CN-STX-CTX-NOR-AMC-CXM-CIP	0(0.0)
13	OFX-CTX-AMC-CXM-TET-CIP-C	0(0.0)
14	STX-OFX-CTX-AMC-CXM-TET-CIP-C	1(2.6)
15	STX-OFX-CTX-NOR-AMC-CXM-TET-CIP	1(2.6)
16	STX-OFX-CTX-NOR-AMC-CXM-CIP-C	2(5.1
17	STX-OFX-CTX-NOR-CXM-TET-CIP-C	1(2.6)
18	STX-CTX-NOR-AMC-CXM-TET-CIP-C	0(0.0)
19	CN-STX-OFX-CTX-NOR-AMC-CXM-CIP	2(5.1)
20	CN-STX-OFX-CTX-AMC-CXM-CIP-C	1(2.6)
21	OFX-CTX-NOR-AMC-CXM-TET-CIP-C	1(2.6)
22	CN-STX-OFX-CTX-NOR-AMC-CXM-CIP-C	2(5.1)
23	CN-STX-OFX-CTX-NOR-AMC-CXM-TET-CIP	0(0.0)
24	CN-STX-OFX-CTX-AMC-CXM-TET-CIP-C	1(2.6)
25	CN-STX-CTX-NOR-AMC-CXM-TET-CIP-C	0(0.0)
26	CN-STX-CTX-NOR-AMC-CXM-TET-CIP-C	1(2.3)
27	STX-OFX-CTX-NOR-AMC-CXM-TET-CIP-C	2(5.1)
28	CN-STX-OFX-CTX-NOR-AMC-CXM-TET-CIP-C	2(5.1)

Key: CN = Gentamicin; STX = Trimethroprim-sulfamethoxazole, OFX = Ofloxacin; CTX = Cefotaxime; NOR = Norfloxacin; AMC = Amoxicillin-Clavulanic acid; CXM = Cefuroxime, TE = Tetracycline, CIP = Ciprofloxacin; C = Chloramphenicol.

Table 5: Multiple Antibiotic Resistance (MAR) Index of *Salmonella typhimurium* isolated from poultry farms in Keffi, Nasarawa state, Nigeria.

No. of antibiotic resistance to (a)	No. of antibiotics tested (b)	MARI Index (a/b)	No. (%) of isolates Feacal droppings (n=19)
10	10	1.0	2(3.6)
9	10	0.9	5(12.8)
8	10	0.8	11(28.2)
7	10	0.7	15(38.5)
6	10	0.6	6(15.4)

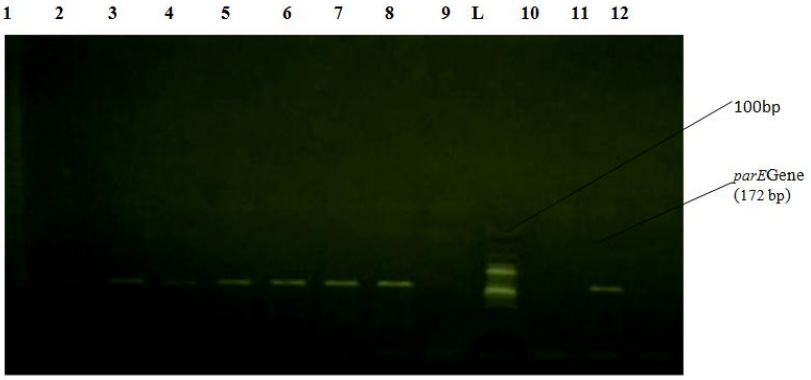

Plate4.1*:* Agarose Gel Electrophoresis of *pare* (172 bp) gene of the *Salmonellatyphimurium*.Lanes2,3,4,5,6,7,8 and 11 represent *pare* Gene bands Lane L represent a 100bp molecularLadder

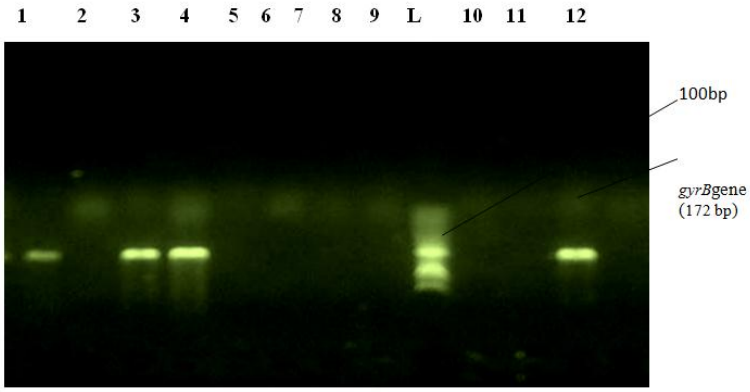

Plate 2*:* Agarose Gel Electrophoresis of *gyrB* (172 bp) gene of the *Salmonella typhimurium*. Lanes 1, 3 4 and 12 represent *gyrB* Gene bands Lane L represent a 100bp molecular Ladder.

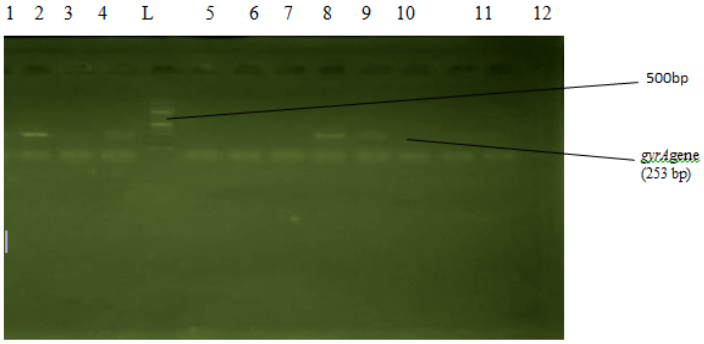

Plate 4.3: Agarose Gel Electrophoresis of *gyrA* (253 bp) gene of the *Salmonella typhimurium*.Lanes24 8 and 9 represent *gyrA* Gene bands Lane L represent a 100bp molecular Ladder

DISCUSSION

The incidence of food borne diseases in human has increased considerably in the last few years. Poultry products have been repeatedly implicated in food borne infections.[1] Over the years, the proportion of *Salmonella typhimurium* with reduced fluoroquinolones susceptibility has increased in many countries of the United Kindom and south East Asia.[14] This focused on molecular detection of plasmid mediated fluoroquinolones resistence genes in ciprofloxacin resistance *Salmonella typhimurium* isolate following almost non availability of such report in this study location the occurrence of *Salmonella typhimurium* from feacal dropping obtained in the study location was expected and is in agreement with the study earlier reported by Tessari *et al.*[15] and Thai *et al*.[16] Out of one hundred and sixty (160) samples obtained, the occurrence of *Salmonella typhimurium* was 24.4%. In this study the occurrence of *Salmonella* isolates was high but lower than a study reported by.[17] in Nsukka Enugu State.

The high occurrence of *Salmonella typhimurium* isolated from the faecal droppings was in agreement with the study early reported by,[16] this implies that the birds are feed with feeds and water that are contaminated with *Salmonella.*

The isolation of *Salmonella typhimurium* in this study indicate the public health significance of the isolates[18] as poultry product contaminated with *Salmonella* may pose serious health hazards.[19,20] The isolation of *Salmonella typhimurium* from poultry farms as observed in this present study could be due to contamination of the feed by *Salmonella* during transportation or storage.[21,22]

The Antibiotic Resistant of *Salmonella typhimurium* isolated from selected poultry farms in respect to feacal droppings were more resistant to Cefotaxime 35 (89.7%), but less resistances to Ofloxacin 12 (30.8%). The high occurrence were not surprising and in this however, justifies their use as common drug of choice for treatment of *Salmonella* infection.[23]

Antibiotic resistant phenotype against *Salmonella typhimurium* isolated from selected poultry farms were distributed into different antibiotic resistance pattern and the most common were STX-CTX-NOR-AMC-CXM-CIP-C with 12.8% Resistance and CTX-NOR-AMC-CXM-TET-CIP-C with 1.03% resistance.

The occurrence of multi drug resistance *Salmonella typhimurium* in the location as was observed in the study not surprising again and this is in agreement with the study earlier describe by.[24] The multidrug resistant (MDR) isolates occurrence was high at 35.5%, this is not in agreement with 94.5%, 47.7%, and 78% reported by.[25,26] respectively in previous studies.

The detection of fluoroquinolone resistant genes namely *parE, gyrB* and *gyrA* in this study is in agreement with the study earlier reported by.[25,26] Our findings in this study shows that *parE, gyrB* and *gyrA* was the main gene detected in ciprofloxacin resistance isolates and this finding is consistence with the study earlier reported by,[27] which reported 66.7% detection of the fluoroquinolone resistant gene observed in the study was indicated that may be responsible for ciprofloxacin resistance.

CONCLUSIONS

The *Salmonella typhimurium* (100%) were resistant to all the antibiotics used in the study namely; amoxicillin clavulanic acid, ciprofloxacin, chloramphenicol, cefuroxine, cefotaxine, gentamicin, norfloxacin, ofloxacin, sulphamethoxazole, trimethroprim, and tetracycline. The *Salmonella* species possess antibiotic resistant genes (gyrA, gyrB and parE) to trimethroprim, chloramphenicol, and tetracycline. The presence of these genes confirms that the isolates were genotypically multi-drug resistant to all the antibiotics employed in the study.

ACKNOWLEDGEMENTS

Special thank you to Ekeleme I Kenneth and Nggada, N. Joshua for their help and support in laboratory work and analysis.

Thank you for your contributions to the study Mr. Dodo K, Ezekiel, Atadoga F. Ojoduwa, in the areas of sample collection and laboratory work.

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