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Molecular Epidemiology of Fluoroquinolones Resistance Genes among Non-typhoidal *Salmonella* Species from Humans and Poultry Faeces in Ido-Ekiti, Ekiti State

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Non-typhoidal *Salmonella* (NTS) strains are among the most common bacterial pathogens associated with antimicrobial resistance (AMR) where animals are known to be the major reservoir of NTS despite improvements in hygiene and sanitation. This study aimed to investigate the prevalence and basis of fluoroquinolone resistance genes in non-typhoidal *Salmonella* (NTS) recovered from human and poultry fecal samples in Ido Ekiti. The study used a total of 300 fecal samples,100 from humans and 200 from poultry sources. Faecal samples were cultured using Selenite F broth and XLD agar. Isolates were identified using morphology, biochemical tests, and molecular characterization. Antibiotic susceptibility were determined using disc diffusion test. Plasmid extraction and curing were done using alkaline lysis and Ethidium Bromide. Phylogenetic

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relatedness was determined using the Unweighted Pair Group Method with Arithmetic Mean. The results revealed that 10.7% of NTS were identified, with 9% from human and 11.5% from poultry sources. High resistance to ofloxacin (77.8%) was observed in both human and poultry samples. S. enteritidis from human sources had a concomitant presence of two qnr genes (qnrA and qnrS) at 20%, while only qnrS (60%) was detected from poultry sources. Statistically significant risk factors for NTS infection among human included the consumption of poultry products and the use of untreated poultry droppings. The study identified Qnr resistance gene in ESBL producing S. enteritidis from both human and poultry sources in Ido Ekiti and are plasmid borne, establishing the role of plasmids in the acquisition, and spread of antibiotic resistance of fluoroquinolones.

Keywords: Non typhoidal salmonella (NTS); fluoroquinolones antibiotics; resistance genes; plasmid genes; feacal samples.

1. INTRODUCTION

The study aims to access prevalence of NTS and the genetic basis of Fluoroquinolone resistance genes in NTS from human and poultry fecal samples in Ido Ekiti and the specific objectives of the study are to: isolate and identify Non Typhoidal *Salmonella* (NTS) species from fecal samples of humans and poultry birds; determine the antibiotic susceptibility profile of NTS to fluoroquinolones; evaluate plasmid profiling and curing of the NTS strains; identify the antibiotic resistant genes in the isolates; characterize the NTS isolates using 16S RNA gene and determine the phylogeny of the NTS strains from both sources.

Antimicrobial resistance (AMR) poses a major public health threat worldwide, and non-typhoidal *Salmonella* (NTS) strains are among the most common bacterial pathogens associated with AMR [1]. In Africa, NTS infections are a significant concern for public health. A study conducted in Ethiopia revealed that NTS was responsible for 33% of all foodborne infections in children under five years old [2]. Although NTS infections can be self-limiting, they may progress rapidly to systemic infection, particularly in infants, older adults, and individuals with weakened immune systems, requiring prompt clinical treatment [3]. The high prevalence of NTS infections in Nigeria is linked to various factors, including poor sanitation, inadequate access to clean water, and inadequate food handling and storage practices [4].

Fluoroquinolone is a commonly used antibiotics for treating NTS infections. However, the increasing prevalence of resistance to antibiotics has become a major challenge in the management of NTS infections [5]. The emergence of resistance to fluoroquinolones in NTS is a major public health concern as it limits treatment options for bacterial infections. Overuse and misuse of these antibiotics has led to the development of resistance in bacteria, which can render them ineffective. Mechanisms of resistance to fluoroquinolones involve mutations in the bacterial DNA gyrase and topoisomerase IV genes, which are the targets of these antibiotics. These mutations prevent fluoroquinolones from binding to bacterial enzymes, thus rendering them ineffective [6]. In addition, bacteria can also develop efflux pumps that can expel antibiotics before they reach their target. This is plasmid-mediated quinolone resistance (PMQR) and involves the acquisition of qnr genes, which encode topoisomerase-binding proteins that confer physical protection against quinolones [7,8].

Plasmid-mediated quinone resistance (PMQR) has played a significant role in the spread of FQ resistance among strains at the human-animal interface. [9] reported that 64.5% of NTS strains were resistant to fluoroquinolones in some parts of Nigeria and a study by [10] found that 36.4% of NTS strains in Ekiti State were resistant to fluoroquinolones, while the WHO lists fluoroquinolones-resistant *Salmonella* spp. as a priority pathogen for the development of new antibiotics in 2017.

2. MATERIALS AND METHODS

2.1 Methods

The Fisher formula was used to calculate the sample size for the study. The sample size was approximately 300 samples for the study. The samples were 200 poultry fecal samples and 100 human fecal sample. The study was conducted between June 2023 and November 2023 at the Federal Teaching Hospital Ido Ekiti (FETHI),

Nigeria. Humans that are immune-compromised such as the patients with HIV, children below 5 and very elderly persons, having gastroenteritis and diarrheal were the selected criteria for the study. Standard procedures were followed to collect clinical fecal samples and poultry fecal samples aseptically.

2.2 Sample Processing

Collected faecal samples in sterile containers within 2hrs of collection were cultured by enrichment in selenite F broth. Using a sterile swab stick, a loopful of inoculum was added to 10 ml of selenite F broth and incubated at 35ºC for 24 hours for incubation. Culture broth containing the sample was streaked onto XLD agar using an inoculating loop and then incubated at 37ºC for 24 hours to confirm growth of the organism [11]. Pure cultures of the isolates were identified based on their cultural, morphological, and microscopic characteristics. Gram staining and biochemical test were carried out according to procedures.

2.3 Antimicrobial Sensitivity Testing

Kirby-Bauer disc diffusion technique was used for antimicrobial sensitivity test and interpreted based on the guidelines of Clinical and Laboratory Standards [12].

2.4 Plasmid Extraction

A basic lysis method was used by [13] for plasmid extraction. The QIAGEN Plasmid Mini Kit was used to detect and isolate plasmids according to the manufacturer's instructions.

2.5 Plasmid Curing of Isolates

Following the method of [14], The isolates containing the target plasmid were cultured overnight at 37^{o}_{C} and 200 rpm overnight for 18 hours by incubating in 1.5ml nutrient broth containing 5ml Ethidium Bromide. The bacterial suspension after cultivation was serial diluted, and 10^{-3} was grown on solid medium. They were thereafter tested for their antibiotic resistance.

2.6 Post Sensitivity Test

Antimicrobial Sensitivity of the bacterial cell after the removal of the plasmid was determined using the Kirby-Bauer disc diffusion technique and interpreted based on the guidelines of the Clinical and Laboratory Standards [12].

2.7 DNA Extraction

DNA was extracted using the TE boil extraction method outlined by [15]. The DNA was afterwards collected by centrifuging it at 13000g for 10 minutes, washed with 500µl of 70% ethanol, air-dried at 37ºC for 3hrs, and finally dissolved in 50µl of TE buffer to solubilize the DNA while protecting it from degradation [16].

2.8 PCR Amplification

The extracted DNA was amplified using multiplex polymerase chain reaction (PCR) assay according to the method described by [11] to detect the presence of the plasmid mediated genes such as qnrA, qnrB, and qnrS for fluoroquinolones that were selected with specific primers:

qnrA(580bp) = F-AGAGGATTTCTCACGCCAGG -R-TGCCAGGCACAGATCTTGAC[11]
qnrS (428bp) = F-ATTTCTCACGCCAGGATTTG -R- GATCGGCAATAGGATAGGTC[10]
qnrB(264bp)=F- ACGACATTCGTCAACTGCAA - R- TAAATTGGCACCCTGTAG[17].

2.9 Sequencing of Purified Genes and Blasting

DNA sequencing of the purified PCR products was performed using BigDye terminator v3.1 cycle sequencing kit according to manufacturer's instructions which generates a DNA sequence readout of the bacterial DNA. MEGA 6 and Bio-Edit software were used to find regions of similarity between biological sequences of the NTS isolates. The software helps to compare nucleotide or protein sequences to sequence databases [18].

2.10 Phylogenetic Analysis

DNA sequences from the PCR product experiments were trimmed by removing all the primer sequences from the ends. They were aligned with the closest match in GenBank obtained by BLAST searches as well as sequences used to design the PCR primers, the joining tress were generated using MEGA UPMGA model [18].

2.11 Statistical Analysis

The data generated from this study were analysed using (SPSS) version 20 (SPSS Inc. Chicago IL) [19].

3. RESULTS

Bacterial morphology showing red-to pink colonies with black center, 1.5-5m in size, odorless, raised, soft but not mucoid, had circular edges, had no pigment, opaque and produces H_2S on XLD agar and colonies that were gram-negative *bacillus* (GNB), positive (+) for catalase test, negative (-) for indole test, negative (-) for citrate test, negative (-) for oxidase test, negative (-) for urease test, positive (+) for motility, produces gas and H2S for Triple sugar iron test were identified as *S. enteriditis.*

NTS was isolated from 9(9.0%) of 100(100%) humans and 23(11.5%) of 200(100%) poultry samples. The overall prevalent rate of NTS was 32(10.7%) of 300(100%) among human and poultry samples.

The antibiotic susceptibility test showed that the highest resistance for the fluoroquinolones antibiotics exhibited by NTS in the human's faecal samples was Ofloxacin 7(77.8%) as compared to Pefloxacin 3(33.3%) as least resistance. The highest resistance for fluoroquinolones antibiotics exhibited by *S. enteriditis* from the poultry's faecal samples was Ciprofloxacin 22(99.6%) as compared to Pefloxacin 16(69.5%) with the least resistance. Interestingly, all strains of NTS isolated from the human and poultry faecal samples were highly resistant to the antibiotics used in the study. The overall results showed a significant difference ($p<0.05$) in the resistance of *S. enteriditis* to the selected antibiotics (Table 1).

Table 1. Overall prevalence of Antibiotics resistance in Non-typhoidal *Salmonella* isolates from human and poultry faecal samples

	Fluoroquinolones Antibiotics			
	CIP	**OFX**	**PEF**	**LEV**
NTS (n)	**(5µg)**	**(5µg)**	**(5µg)**	**(5µg)**
Human (n=9)	5(55.6%)	7(77.8%)	3(33.3%)	4(44.4%)
Poultry(n=23)	22(95.6%)	19(82.6%)	16(69.5%)	20(86.9%)

CIP=Ciprofloxacin, OFX=Ofloxacin, PEF=Pefloxacin, LEV=Levofloxacin.
N= Number of positive NTS, %;= Percentage

Table 2. Prevalence of positive NTS in relation to demographics

	Positive(%)	**Pvalue**	**x^2**	**df**
Age				
1-10	3 (33.3%)			
11-20	1(11.1%)			
61-70	5 (55.5%)	0.041	0.034	1
Total	9 (100%)			
Sex				
Male	3 (33.3%)			
Female	6 (66.6%)	0.485	4.46	2
Total	9 (100%)			
Occupation				
Trader	2 (22.2%)			
Farmer	4 (44.4%)	0.300	1.073	1
None	3 (33.3%)			
Total	9 (100%)			
Educational				
Primary	5(55.5%)	0.09	6.760	1
Tertiary	1(11.1%)			
None	3(33.3%)			
Total	9 (100%)			
Marital status				
Married	7 (77.7%)	0.11	0.716	3
Single	2 (22.2%)			
Total	9(100%)			

Table 3. Risk factors of patients with NTS

Risk factors	No of Positive (%)
Consumption of vegetables grown with manure	7 (7%)
Consumption of Poultry products (Eggs, chicken meat)	9 (9%)
Old age (> 60 years)	5 (5%)
Well water only	4 (4%)
Well/Tap water	9 (9%)
History of NTS	1(1%)
Family history of NTS	0

Plate 2 shows the electrophoresis gel of the selected Ten NTS isolates with 16S primer. Lane M is the Molecular marker, lanes 1-5 are *S. enteritidis* (poultry) and Lane 6-10 are *S. enteritidis* (humans). It also showed that all the isolates had 16s rRNA with a molecular weight of 1500bp.

The plasmid analysis of the ten selected NTS isolates in both patients and poultry fecal samples is represented in Plate 1. *S. enteritidis on* Lane 1-5 are from patients while lane 6-10 are *S. enteritidis* from poultry samples. The plasmid DNA with 5000bp was shown to be present in all selected NTS isolates.

The pre and post-curing antibiotic susceptibility tests of the ten selected isolates with antibiotic resistances from human and poultry faecal samples was observed that before plasmid curing, all isolates were resistant to all the antibiotics used in this study, and after post curing, all isolates were now sensitive to the drugs to which they had formally shown resistance. This confirms that all isolates selected for the study were plasmid mediated.

Plate 3 shows the detection of Fluoroquinolones genes *QnrA, Qnr S, Qnr B* from NTS by multiplex polymerase chain reaction (PCR) with molecular weight of 580 bp, 428bp and 264bp respectively while using specific Primers. It was revealed that Lanes 1-5 are *S. enteritidis* from poultry while Lanes 6-10 are S. *enteritidis* from human, Lane 11 is a buffer. QnrA was found to be present in two poultry isolates (FP3, DP4) and one human Isolate (H32), QnrB was found present in a poultry isolate (KP3) and QnrS was found in three poultry isolates (OP5, FP3, KP6) and one human isolate (H10). It detected that isolate FP3 has both QnrA and QnrS genes present in it and isolate KP3 has both QnrS and QnrB present in them.

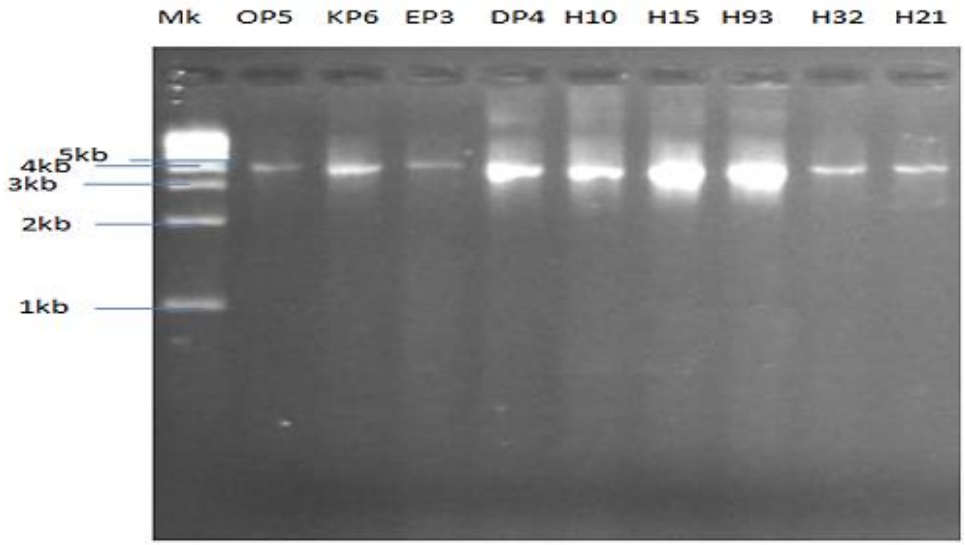

Plate 1. Plasmid DNA of Non-typhoidal Salmonella on 1 % agarose. Mk-Molecular markers, Lanes DP5, Kp6, EP3, DP4=NTS from Poultry, Lanes H10, H15, H93, H32, H21-NTS from patients

Table 4. Pre and post -plasmid curing antibiogram of the Non-typhoidal *Salmonella* from human and poultry faecal samples

ID	CIP (5µg)		OFX (5µg)		PEF (5µg)		LEV (5µg)	
	PRE	POST	PRE	POST	PRE	POST	PRE	POST
OP6	R	S	R	S	R	S	R	S
KP6	R	S	R	S	R	S	R	S
EP6	R	S	R	S	R	S	R	S
DP4	R	S	R	S	R	S	R	S
H10	R	S	R	S	R	S	R	S
H15	R	S	R	S	R	S	R	S
H93	R	S	R	S	R	S	R	S
H32	R	S	R	S	R	S	R	S
H21	R	S	R	S	R	S	R	S

KEY= CIP=Ciprofloxacin, OFX=Ofloxacin, PEF=Pefloxacin, LEV=Levofloxacin, R: RESISTANT, S: SENSITIVE (CLSI, 2020)

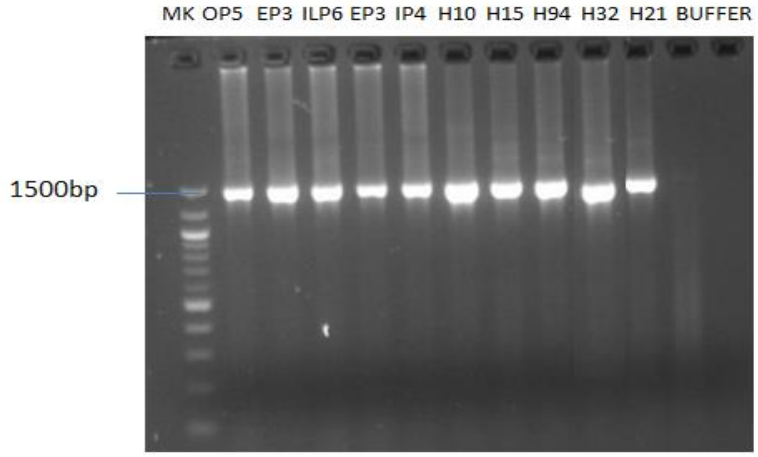

Plate 2. Electrophoresis gel of the NTS isolates with 16S primer (bp= 1500)
Lane Mk= Molecular marker, Lane 1-5= S. enteritidis (poultry), Lane 6-10= S. enteritidis(humans)

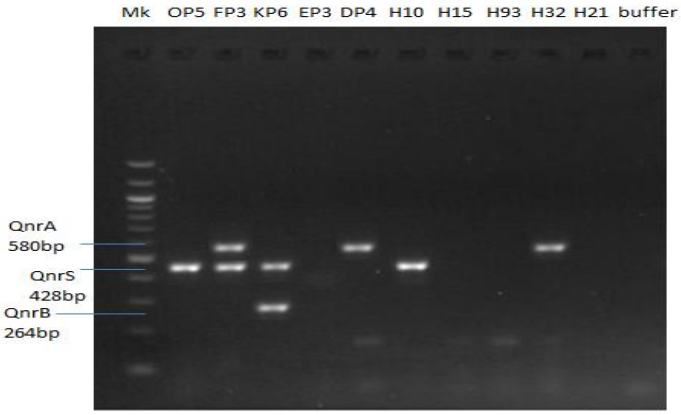

Plate 3. Detection of Fluoroquinolones genes *QnrA, Qnr S, Qnr B* by polymerase chain reaction (PCR)

QnrA,= F- AGAGGATTTCTCACGCCAGG R- TGCCAGGCACAGATCTTGAC
Qnr S= F- ATTTCTCACGCCAGGATTTG R- GATCGGCAATAGGATAGGTCA
Qnr B= F- ACGACATTCGTCAACTGCAA R- TAAATTGGCACCCTGTAGGC
M=Molecular markers, Lanes= OP5, Kp6, EP3, DP4= NTS from Poultry, Lanes H10, H15, H93, H3, H15=NTS from patient

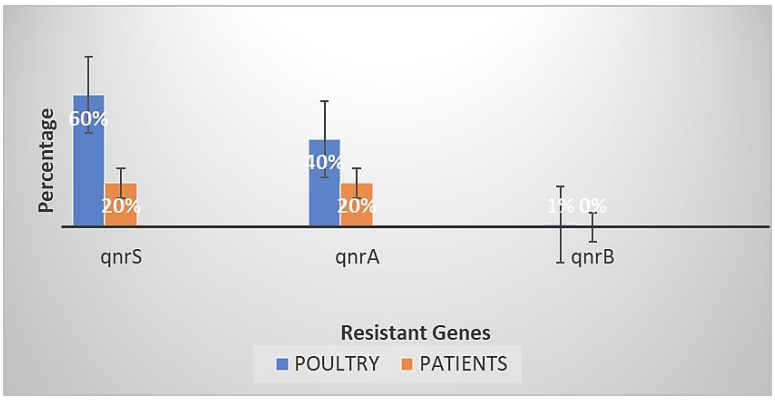

Fig. 1. Percentage distribution of resistance genes among isolated S. enteritidis

Table 5. Blasting sequence of the Non-typhoidal *Salmonella* strains

Sample ID	Strain	Source	Accession	Percentage Similarity
OP5	*Salmonella enteritidis* 04-7505	Poultry	OQ048684	100%
FP3	*Salmonella enteritidis* 04-7052	Poultry	OQ048685	100%
KP6	*Salmonella enteritidis* 04-6191	Poultry	OQ048686	100%
EP3	*Salmonella enteritidis* 04-6189	Poultry	OQ048687	100%
DP4	*Salmonella enteritidis* 04-7529	Poultry	OQ048688	100%
H10	*Salmonella enteritidis* 05-6746	Human	OQ048689	99.99%
H15	*Salmonella enteritidis* 05-6557	Human	OQ048690	99.99%
H93	*Salmonella enteritidis* 05-1219	Human	OQ048691	99.99%
H32	*Salmonella enteritidis* 05-6733	Human	OQ048692	99.99%
H21	*Salmonella enteritidis* 06-1751	Human	OQ048693	99.99%

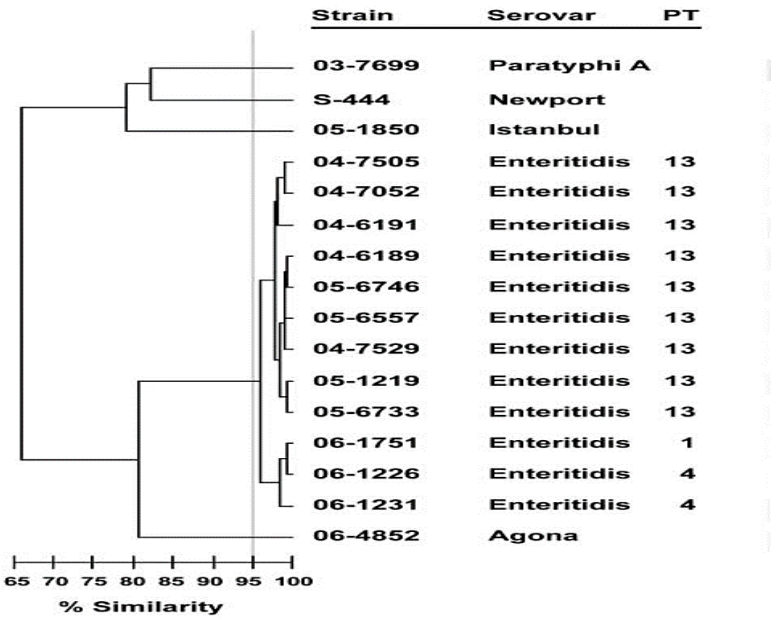

Fig. 2. Phylogenic tree of selected Non typhoidal *Salmonella* from human and poultry feaces

Key: Lane 4- S.enteritidis (kp4) ; Lane 5- S.enteritidis (EP3);
Lane 6- S.enteritidis (OP5) ; Lane 7- S.enteritidis (FP3);
Lane 8- S.enteritidis (Dp4) ; Lane 9- S.enteritidis (H93);
Lane 10- S.enteritidis (H10) ; Lane 11- S.enteritidis (H21);
Lane 12- S.enteritidis (H15) ; Lane 13- S.enteritidis (H32);

Fig. 1 shows the distribution of resistance genes among *S. enteritidis* isolated from patients and poultry faecal samples. It showed that 10 selected isolates were tested with three resistance genes (*QnrA, Qnr S, Qnr B*). For human isolates, *qnrS and qnrA* gene were only detected in 20% of the isolates respectively with *qnrB* gene showing no presence in the isolates as compared to poultry samples where *qnrS and qnrA* gene were only detected in 60% and 40% of the Isolates respectively with *qnrB* gene showing no presence in the isolates.The total percentage gene occurrence amongst the 10 selected isolates from human and poultry faecal isolates showed *qnrS* and *qnrA* gene with total percentage occurrence of 4(40%) and 3(30.0%) respectively for Fluoroquinolones genes. This confirms the transfer of *S. enteritidis* from poultry sources to human sources because of its high percentage occurrence.

Table 5 shows the result of the sequencing of the Non typhoidal *Salmonella.* It revealed that all the *Salmonella enteritidis* identified by blasting were 100% and 99.9% related to some of the non-typhoidal *Salmonella* species in the gene bank.

Fig. 2 shows the result of the phylogenic tree of ten selected Non typhoidal *Salmonella* strains from human and poultry faeces. The dendogram constructed using the UPMGA method was a well rooted tree with *S.Agona* used to create an outgroup that showed fifteen different clusters. This revealed that *Salmonella S. enteritidis* from the study serovars have a similarity matrix of 94.8% which shows that they are related, it also revealed that other *Salmonella* serovars show a close relationship to the study serovars have having similarity matrix of 81.7% which shows that they are closely related.

4. DISCUSSION

Non-typhoidal *Salmonella (*NTS) is a very important pathogenic organism that its prevalence shows a serious threat to man and the isolation from poultry and clinical sources has not received much attention in Ekiti State, Nigeria [20]. However, the aim of this study was to isolate NTS from poultry and human' faecal samples in Ido Ekiti, and poultry is confirmed to remain one of the major routes of transmission of this organism. The results obtained showed varying frequencies in the isolation of non-typhoidal *Salmonella* in both hᵘmans and poultry in this study. Nine out of one hundred immunocompromised patients had NTS. Many studies have shown that Non-typhoidal *Salmonella* (NTS) is among the very common pathogens causing bacterial bloodstream and gastroenteritis in adults and children in sub-Saharan Africa [21,22]. Twenty-three out of two hundred poultry samples had NTS and the rate of isolation is higher compared to the isolation from humans. The prevalence of NTS from human faeces was 9.0% and poultry fecal sample had an 11.5% isolation rate. This agrees with previous work of culture-based studies of diarrhea in sub-Saharan Africa by [17] who reported that non-typhoidal *salmonella*e are isolated in 2–27% of stool culture-positive diarrhoeal illness.

The prevalence of the NTS among poultry faecal was 13 (56%) which was statistically significant (P value 0.05) and confirms it as the main source of spread to humans. This agrees with [23] who reported a 64 (6.4%) prevalence rate of NTS among 100 samples collected from poultry. This is because Non Typhiodal *Salmonella* species are prevalent and they occur as pathogenic bacteria in the intestines of domestic, poultry, and wild animals. This also agreeds with CDC (2000) which reported that the prevalence of NTS among chickens in Michigan was 9.2%. Nevertheless, this might pose a major pressure on the spread of NTS to humans since humans and warm-blooded animals are the prime reservoirs of non-typhoidal *Salmonella* and subsequent shedding can result in the spread to humans and their environs [24]. This also agreed with [25] who reported that Non-typhoidal *Salmonella* infections are generally allied with the consumption of contaminated food products from poultry or ruminants.

Antibiotic resistance of NTS isolates from sick humans and poultry faeces as shown in the table may be because poultry birds are usually given antibiotics which have created antibiotic resistance in humans [26]. There have been reports indicating that the use of antimicrobials for growth promotion, prophylaxis, and treatment of food animals can increase the prevalence of resistance in human pathogens, particularly non-typhoidal *Salmonellosis*. This practice is common in Asia, the United Kingdom, and some other European countries [27] where fluoroquinolones have been approved for animal use. [23] reported on the recent ban on the importation of poultry products into Nigeria that could have led to an increase in smuggling, which may be a likely reason for the emergence of resistance to fluoroquinolones.

Plasmid has been documented to have an encoded gene that provides resistance to naturally occurring antibiotics in competitive environmental niches [28,29]. Although not all the NTS carry plasmid, for the NTS that carries plasmid the resistances are always more and the resistance of NTS that are not due to plasmid might be chromosomal which may be a mutation of genes encoding ribosomal protein which decreases the permeability of the cell envelop in enteric bacteria [30]. This study confirms that resistance in NTS was all plasmid-mediated. This is backed by [31] who reported that a plasmid-mediated mechanism could increase the likelihood of horizontal spread.

The NTS was tested with three different resistant genes of Non-typhoidal *Salmonella*. This research verifies that the *Salmonella* isolates collected from both poultry and human' faecal samples carry the same resistance genes. These genes may be crucial for the bacteria's ability to invade and survive in the host. This aligns with a previous study by [23], which found that NTS isolates from both humans and animals exhibited similar resistance genes. This indicates a possible danger for humans who consume poultry products. This study identified three qnr gene groups (qnrA, qnrB, and qnrS), with qnrS being the most predominant at 40%, followed by qnrA at 30%; qnrB was not present amongst the isolates. The frequencies of these genes were higher than those reported in Niger, where the qnr genes were 32.3% for qnrS and 26.2% for qnrA, according to Moumouni [32] study. These genes are typically plasmid-mediated and can quickly spread among the members of Enterobacteriaceae through gene transfer mechanisms [33,35]. Several studies have shown that the qnr gene responsible for quinolone resistance is often carried by high molecular weight conjugative plasmids. This means that the gene can be transferred between bacteria, leading to the widespread rise of quinolone resistance through horizontal gene transfer. Researchers like [35,36,37] have all documented these findings.

5. CONCLUSION

The results of the study helped to explain the molecular prevalence of NTS in human and poultry faeces in Ido Ekiti and the study confirmed that poultry in Ido Ekiti might be a major source of resistant NTS because of its high prevalence in the study area, and this poses a potential hazard to the public, especially those living close to the poultry and those rearing them. Also, the resistance to antibiotics in this study was confirmed to be only plasmid-mediated. The study results indicated a high occurrence of qnrA and qnrS genes, Also, the molecular relatedness of the NTS isolates in humans and poultry suggested that there might be a likelihood of the spread of these resistance strains from poultry to humans in this study environment. These finding thus suggests the strengthening of the public health policies in Nigeria to prevent, monitor, and control antimicrobial resistance via the implementation of an antibiotic resistance surveillance system.

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CONSENT

A questionnaire to obtain demographic characteristics and other relevant information to the study was given to the participant. Written consent for participation in the study was obtained from the participants.

ETHICAL APPROVAL

Ethical clearance for the study was obtained from the Ethical committee of the Federal Teaching hospital, Ido Ekiti after due processes with an ethical certificate no ERC/2022/06/06/794A.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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