

Detection of Plasmid-borne *qnr* Genes in Quinolone-Resistant *Salmonella typhi* from Patients Attending National Hospital, Abuja, Nigeria.

ABSTRACT

Aims: This study aimed to investigate and report the presence of the *qnr* genes among *S. typhi* isolated from the stool of patients with suspected typhoid fever, at National Hospital, Abuja, Nigeria

Study design: Cross sectional study

Place and Duration of Study: Department of Microbiology, Nasarawa State University, Keffi, between October 2020 and April 2022.

Methodology: Stool samples of patients with suspected typhoid fever were collected by standard methods in sterile disposable containers. After analysis of stool, microscopic observations and culture analysis, *Salmonella typhi* was isolated, antibiotic susceptibility testing was carried out on the isolate, and the bacterial genome was extracted by boiling method. PCR for detection of *qnr* genes including *qnrA*, *qnrB* and *qnrS* was done by specific primers, then PCR products were run using gel electrophoresis and visualized by gel documentation system.

Results: 6.6% of samples isolated (10/150) were positive for *S. typhi*. Antibiotic resistance among the isolates in decreasing order were as follows: imipenems (100.0%), cefuroxime (100.0%), cefotaxime (100.0%), nalidixic acid (90.0%), amoxicillin/ clavulanic acid (90.0%), levofloxacin (80.0%), ceftriaxone/sulbactam (70.0%), ciprofloxacin (70.0%), gentamicin (70.0%), and ofloxacin (60.0%). The most common antibiotic resistant phenotype was AUG- CTX-IMP-OFX-CN-NA-CXM-CRO-CIP-LBC at 20.0%. Multiple antibiotic resistance (MAR) was observed in 100% (10/10) of the isolates with the common MAR indices being 0.8 and 0.9 (30.0%). The only positive *PMQR* genes were *qnrA* and *aac(6)-Ib-cr*.

Conclusion: The *S. typhi* isolates showed high resistance to all the isolates, and all isolates were MAR, with resistance to 9 and 8 antibiotics being the most predominant. In addition, *qnrA* resistance gene was the most common gene expressed.

Keywords: *Salmonella typhi*; Stool; antibiotics; Resistance; *qnrA*.

1. INTRODUCTION

Salmonella enterica serovar Typhi (*S. Typhi*), the causative agent of typhoid fever, which is a common human-specific pathogen, has been widely acknowledged as the primary cause of mortality resulting from enteric pathogen infections (1,2). The Organism is a Gram-negative, rod shape, flagellated and aerobic bacteria; it is posing a great danger to human health, most notably in low- and middle-income countries (3). Every year, there are 21 million reported cases of typhoid fever, resulting in 200,000 fatalities globally (3). For a long time, the medication for Salmonellosis infections were Penicillin, Chloramphenicol and trimethoprim. An increase in resistance to such antibiotics and many more drugs led to the emergence of newer antibiotics, known as the Fluoroquinolone groups (4). The unresponsiveness of Fluoroquinolones are due two mechanisms: chromosomally mediated mutations in topoisomerase's quinolone resistance determining regions (QRDR) and quinolone resistance determining region mutations; resistance genes belong to *qnr* groups plasmids mediated play a role in Fluoroquinolone resistant (5). Fluoroquinolone resistance is conferred by both chromosomal and plasmid- encoded fluoroquinolone resistance (6). Determinants belonging to *qnr* have been found in a numeral of enterobacterial species from different parts of the world, including Asia, America, Europe and Africa (7, 8). So far, six variants (*qnrA1* to *qnrA6*) have been discovered. Quinolones produced by other plasmids *qnrB* (*qnrB1* to *qnrB5*) and *qnrS* (*qnrS1* and *qnrS2*) resistance determinants outlined in enterobacterial species (9). The current study aimed to investigate the presence of *qnrA*, *qnrB* and *qnrS* genes in quinolone-resistant *Salmonella typhi* (*S. typhi*) isolated from stool samples of patients with suspected typhoid fever in the National Hospital, Abuja, Nigeria.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Media

Bacteriological media that were used in this study include: Salmonella-Shigella (SSA) Agar; Nutrient agar (NA); Mueller-Hinton agar (MHA); Mueller-Hinton broth (MHB); Bismuth sulfite agar (BSA); Selenite F- Broth (SFB); Xylose Lysine Deoxychocolate agar (XLD); Simmons Citrate agar (SCA); Triple Sugar Iron agar (TSI); and Peptone water (PW).

2.1.2 Equipment

The equipment used in this study include an autoclave, Oven, Incubator, Refrigerator/Freezer, Thermocycler, Gel electrophoresis machine, Laminar air flow cabinet, Microscope, Spectrophotometer, UV illuminator, Centrifuge, Touch plate Super Mixer, Microwave oven, Electronic weighing balance, Vortex machine and Gel Doc system.

2.1.3 Chemicals and reagents

The chemicals and reagents that were used in this study include: Acridine orange, Carbofuschin, Crystal violet, Ethanol, Xylene solution, Creatinine, Potassium hydroxide, Kovac's reagents, Ethydium bromide, Iodine solution, EDTA and Glycerol

2.1.4 Bacteria Isolates

Confirmed *S. typhi* isolates from the stool of patients were obtained and used for this study.

2.1.5 Study Location

The study was carried out at the National Hospital, Central Business District, Abuja (NHA). NHA is a 400 - bed hospital (Secondary health facility) that renders specialist services in all areas of medicine, located in Garki, within the Federal Capital Territory of Nigeria.

2.2 Methods

2.2.1 Sample Collection

A total of 150 stool samples of patients with suspected cases of typhoid fever were collected using sterile container and transported using icepack to the Microbiology Laboratory, Nasarawa State University, Keffi for analysis.

2.2.2 Isolation and Identification of *Salmonella typhi*

Salmonella typhi was isolated and identified using Gram staining, indole test, methyl red test, Voges-Proskauer test, citrate test and oxidase test as described by [10] and further identified using KB003HI25 TM identification kits following the manufacturer's instruction.

2.2.3 Antibiotic Susceptibility Testing

The antibiotic susceptibility test of the isolates was carried out using the Kirby-Bauer disc diffusion method as modified by the Clinical and Laboratory Standards Institute - CLSI [10]. Briefly, (3) pure colonies of the isolate from stool samples of patients in the selected hospital was inoculated into 5 ml sterile 0.85% (w/v) NaCl (normal saline) and the turbidity of the bacteria suspension was adjusted to the turbidity equivalent to 0.5 McFarland standard. The McFarland's standard was prepared as follows; 0.5 ml of 1.172% (w/v) BaCl₂·2H₂O was added into 99.5 ml of 1% (w/v) H₂SO₄ [11]. A sterile swab stick was soaked in the standardized bacteria suspension and streaked on the Mueller-Hinton agar plates and the antibiotics disc were aseptically placed at the centre of the plates and allowed to incubate at room temperature for 1 hour before re-incubating at 37°C for 17 hours. The discs used include: Gentamicin (CN: 10 µg), Amoxicillin/Clavulanate (AMC: 30µg), Cefotaxime (CTX: 25 µg), Imipenem/Cilastatin (IMP: 10 µg), Ofloxacin (OFX: 5 µg), Nalidixic acid (NA: 30 µg), Cefuroxime (CXM: 30 µg), Ceftriaxone/Sulbactam (CRO: 30 µg), Ciprofloxacin (CIP: 5 µg), and Levofloxacin (LBC: 5 µg). After incubation, the diameters of the zones of inhibition were measured to the nearest millimeter (mm) using a ruler and the result of the susceptibility test was interpreted using susceptibility breakpoint earlier described by CLSI [10].

2.2.4 Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR index of the isolates was determined as described previously [12] using the formula:

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

2.3 Molecular detection of Quinolone resistance genes

2.3.1 DNA extraction

The bacterial DNA was extracted by a method described by Abimikuet *al* [13] with minor modification. Ten (10) milliliters of an overnight broth culture of the bacterial isolate in 1 ml Luria-Bertani (LB) were spun at 14000 rpm for 3 minutes. The supernatant was discarded, and the harvested cell pellet was re-suspended in 1 ml sterile distilled water and transferred into 1.5 ml centrifuge tube and centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded carefully. The pellet was re-suspended in 100 µl of sterile distilled water by vortexing. The tube was centrifuged again at 14000 g for 10 minutes, and the supernatant was discarded carefully. The cells were re-suspended in 500 µl of normal saline and heated at 95°C for 20 minutes. The heated bacterial suspension was cooled on ice for 10 minutes and spun for 3 minutes at 14000 rpm. The supernatant containing the DNA was transferred to a 1.5-ml microcentrifuge tube and stored at -20°C for other reactions.

2.3.2 Amplification of Target Genes

The DNA amplification of target plasmid-mediated Quinolone resistant genes in ciprofloxacin resistant *S. typhi* isolates was carried out using single plex method by modification of the method earlier described by [14]. Briefly, the reaction was carried out in 25 µl reaction volume in artificial tubes which is made up of 5 µl master mix, 2.4 µl primers (0.4 µl each of forward and reverse primers), 0.5 µl of MgCl₂, 1.5 µl of DNA template and

15.6 µl of nuclease free water. The reaction tubes were placed in the holes of the thermal cycler was closed and the door was closed. Then *qnrA*, *qnrB*, and *qnrS* genes were amplified under the following conditions: Initial denaturation at 94°C for 5 min followed by 32 cycles of amplification at 94°C for 45 sec each, annealing at 53°C for 45 sec, with final extension at 72°C for 5 min [14].

The amplification condition for detection of *aac (6) -1b-cr* was carried out as follows; initial denaturation at 95°C for 20 min, annealing at 59°C for 40 sec and initial extension at 70°C for 30 sec and with final extension at 72°C for 5 min [14]. The primers used are listed in Table 1.

2.3.3 Agarose gel electrophoresis

The PCR products (10 µl) were evaluated on a 1.5% (w/v) Agarose gel (Gibco Life Technologies, Paisley, United Kingdom) at 100 mV for 60 min using BIO-RAD Power Pac 3000; and a molecular weight marker (1-kb DNA Ladder) was used as a standard. The DNA bands were then visualized and photographed under UV light using UVitec and Video copy processor after staining the gel with ethidium bromide as described by [15].

Table 1. Primers and their sequences

Target gene	Primer Sequence	Annealing Temperature (°C)	Amplicon size (bp)	Reference
<i>qnrA</i>	5'- CCGCTTTTATCAGTGTGACT -5' 3'- ACTCTATGCCAAAGCAGTTG -3'	5 5	188	[16]
<i>qnrB</i>	5'- GATCGTGAAAGCCAGAAAG G -5'3'- ACGATGCCTGGTAGTTGTCC -3'	5 4	469	[16]
<i>qnrC</i>	5'- GGGTTGTACATTTATTGAATC G -5'3'- CACCTACCCATTTATTTTCA - 3'	5 4	308	[16]
<i>qnrD</i>	5'- CGAGATCAATTTACGGGGAAT A-5' 3'-AACAAAGCTGAAGCGCCTG - 3'	5 7	582	[16]
<i>QnrS</i>	5'- ACGACATTCGTCAACTGCA A- 5' 3'- TAAATTGGCACCCCTGTAGG C- 3'	5 5	417	[16]
<i>aac(6')-1b</i>	5'- TTGCGATGCTCTATGAGTGGCT A-5' 3'-	5 7	482	[16]

	CTCGAATGCCTGGCGTGTTT-3'			
Class1	5'-TCCACGCATCGTCAGGC -5'	5	280	[16]
Integron	3'-CCTCCCGCACGATGATC-3'	5		

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of *Salmonella typhi*

The organism which grew with colorless colonies on Salmonella-Shigella (SSA) Agar, black metallic sheen on Bismuth Sulphite Agar, Gram negative, rod shape, nitrate-positive, Hydrogen sulphide-positive, and Methyl red-positive was identified as *S. typhi*.

3.2 Occurrence of *Salmonella typhi*

The occurrence of *S. typhi* was 6.7 % (10/150). In relation to age of patients, the occurrence of *S. typhi* was highest at age >50yrs (17.6%), but lowest in age ≤ 10 (0.0%) as shown in Table 2.

Table 2. Age Distribution of the Study Population

Age	No of Samples	Number (%) <i>S. typhi</i>
≤ 10	15	0(0.0)
11- 20	18	1(5.6)
21- 30	35	3(8.6)
31- 40	35	2(5.7)
41- 50	30	1(3.3)
> 50	17	3(17.6)
Total	150	10(6.7)

3.3 Antimicrobial Resistance Profile

The Antibiotic Resistance of *S. typhi* isolates from patients with suspected typhoid fever in University of National Hospital, Abuja, is as given in Table 3. The isolates from NHA were totally resistant to Cefotaxime (100.0%), imipenem (100.0%), and cefuroxime (100.0%), with all other antibiotics having a resistance percentage not less than 60%.

Table 3. Antimicrobial resistance profile of *S. typhi* from the stool of patients with suspected typhoid fever in National Hospital, Abuja, Nigeria

Antibiotics	Disc Content (µg)	No. (%) resistance in <i>S. typhi</i> (n=10)
Amoxicillin/Clavulanate (AMC)	30	9(90.0)
Cefotaxime (CTX)	25	10(100.0)
Imipenem/Cilastatin (IMP)	10	10(100.0)
Ofloxacin (OFX)	5	6(60.0)

Gentamicin (CN)	10	7(70.0)
Nalidixic acid (NA)	30	9(90.0)
Cefuroxime (CXM)	30	10(100.0)
Ceftriaxone/Sulbactam (CRO)	30	7(70.0)
Ciprofloxacin (CIP)	5	7(70.0)
Levofloxacin (LBC)	5	8(80.0)

3.4 Multiple Antibiotic Resistance (MAR) Index

Multiple antibiotic resistance (MAR), which is the resistance of microorganisms to at least two (2) antibiotics was observed in all the isolates. The commonest indices were 0.9 (30.0%), 0.8 (30.0%), and 1.0 (20.0%) as shown in Table 4.

3.5 Molecular Detection of Plasmid Mediated Quinolone resistant genes

The plasmid mediated quinolone resistant genes detected in quinolone resistant isolates is as shown in table 5. The only positive *PMQR* genes were *qnrA* and *aac(6)-Ib-cr*.

Table 4. . Multiple Antibiotics Resistance (MAR) Index of *Salmonella typhi* isolates from stool of patients from National Hospital, Abuja, Nigeria

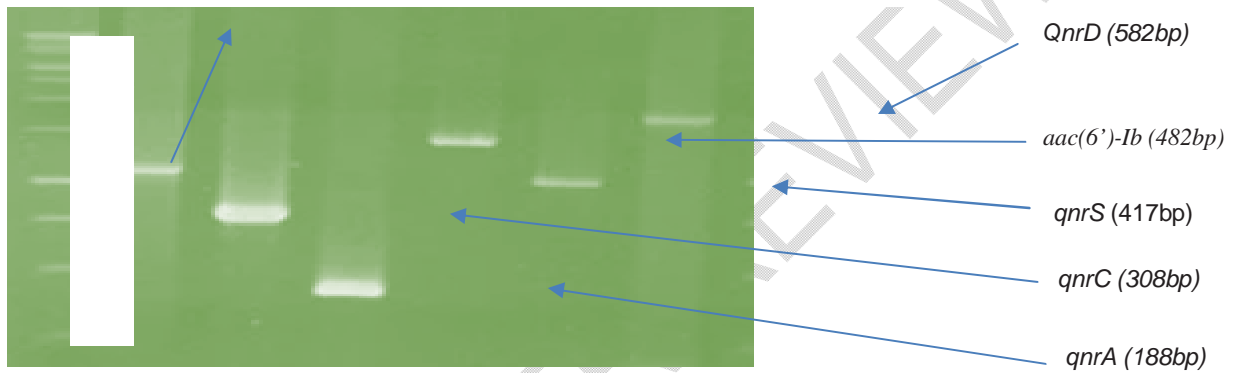
No. of Antibiotic Resistance (a)	No Antibiotics Tested (b)	of MAR Index (a/b)	No (%) of MAR Isolates (n =17)
10	10	1.0	2(20.0)
9	10	0.9	3(30.0)
8	10	0.8	3(30.0)
7	10	0.7	1(10.0)
6	10	0.6	0(0.0)
5	10	0.5	1(10.0)
4	10	0.4	0(0.0)

Table 5. Molecular Detection of plasmid mediated Quinolone Resistance genes in Quinolone resistant *Salmonella typhi* National Hospital, Abuja, Nigeria.

Quinolone resistance Genes	No. (%) of <i>S. typhi</i> (n = 1)
<i>QnrA</i>	1(100.0)
<i>QnrC</i>	0 (0.0)
<i>QnrD</i>	0 (0.0)
<i>QnrS</i>	0 (0.0)

<i>aac(6)-Ib-cr</i>	0 (0.0)
<i>qnrA+qnrC</i>	1 (100.0)
<i>qnrC + qnrD</i>	0 (0.0)
<i>qnrD + qnrS</i>	0 (0.0)
<i>qnrS + aac(6)-Ib-cr</i>	0 (0.0)
<i>qnrA + qnrC + qnrD +qnrS + aac(6)-Ib-cr</i>	1 (100.0)

*MAR isolates are those with resistance to at least two antibiotics [15]



3.6 Discussion

Salmonella typhi, which is known for being resistant to several antibiotics is the causative agent for Typhoid fever. It is a major health worry as it causes of death and infections in many developing nations [17], as well as illnesses in Africa, particularly Nigeria. In our study, 10(6.7%) identified as *S. typhi*. This percentage is lower than 13% reported in a study by Jubair *et al* in Iraq [18], 47% reported by Jabeen *et al* in Pakistan [19], and 80% in Middle Eastern countries reported in a study by Rahman *et al* [20].

The occurrence of *S. typhi* from the stool of patients in relationship to their age was higher at age > 50 years (17.6%), closely followed by age 21-30 years (8.6%). These figures differ from reports in studies by Jubair *et al* in Iraq [18], Aljanabyet *al* in Iraq [21] and Safdar *et al* in Pakistan [22], where age 21-30 years had a higher occurrence 15.8%, 16.2% and 16.7% respectively. The higher rates of occurrence among adults in these studies might be due to exposure and lack of good hygiene especially with food.

The high rate of resistance of the isolates to antibiotics such as cefuroxime, cefotaxime, imipenem, amoxicillin/clavulanate, and nalidixic acid (all above 90%) as observed in this study is probably due to the abuse and misuse of antibiotics. The isolates were highly resistant to

cefuroxime, cefotaxime and imipenem (all 100%). This was in contrast with findings from a study in Iraq by Njumet *al* [23], where isolates were sensitive (100%) for ceftriaxone, cefepime, cefazolin and chloramphenicol. Furthermore, similar resistance to antibiotics including Ofloxacin(60.0%), gentamicin(70.0%), and ciprofloxacin(70.0%) was observed in a study by Njumet *al* and Namratha *et al.*,2015 [24]. In Iraq and India respectively. including The resistance of these isolates to antibiotics means they could be presented as the best option for treatment of infections caused by *S. typhi* in this region.

The Multiple antibiotic resistance (MAR), which is the resistance of microorganisms to at least two (2) antibiotics was observed in all 15 isolates (100.0%). The three most common PMQR gene. (*qnrA*, *qnrB*, and *qnrS*) are known to lead and cause the spread of quinolone-resistant *S.typhi*, and they were chosen for use in this study. However, the only positive PMQR genes were *qnrA* and *aac(6)-Ib-cr*with percentage occurrence of 100.0% respectively. In South Korea, the *qnrB* gene was amplified in 70.0% of *S.typhi* strains isolated [25]. In Iran, *qnrA* was found in 30.4% of the *S.typhi* isolates, *qnrS* was detected in over 50.0%, while *qnrB* was detected in 1.1% [24]. A study on *qnr* genes in *S.typhi* isolates from India found that *qnrB* was the most common *qnr* gene [26].

4. CONCLUSION

This study demonstrated that cephalosporins (ceftriaxone, cefuroxime and cefotaxime) and carbapenems (imipenem), are fully effective against *S.typhi* clinical isolates from typhoid fever patients. The findings of this study report the presence of plasmid-mediated quinolones-resistant genes in *S.typhi* clinical isolates from patients with suspected typhoid fever in Abuja, Nigeria. The *qnrA* gene was the visible gene. Resistance genes may contribute to the quinolone resistance of *S. typhi*. With the appearance of plasmid-mediated quinolones-resistant genes in *S.typhi*, it's important to limit the use of quinolones and have good infection control to keep resistant strains from spreading.

CONSENT

All authors declare that 'written informed consent was obtained from the patient approved parties for publication of this study. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

Appropriate ethical committee approval was obtained prior to the start of the research and is available for review (Attached to this publication draft)

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