Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i3.5078

A MOLECULAR ANALYSIS: DETECTION OF PLASMID-BORNE *QNR* GENES IN QUINOLONE-RESISTANT *SALMONELLA TYPHI* ISOLATES FROM PATIENTS AT A TERTIARY CARE HOSPITAL IN BAHAWALPUR, PAKISTAN

Safdar Ali[1*], Muhammad Rizwan[2*], Maria Rasool[3], Rafia Anwar[3, 4], Maimona Sadia[4], Irfan Ahmad[5], Areej Safdar[6], Laiba Qadir[7]

[1*]Faculty of Science and Technology, Department of Molecular Biology, Virtual University of Pakistan
[2*,7]Department of Microbiology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan
[3]College of Allied Health Professionals, Government College University Faisalabad, Pakistan
[3,4,4]Department of Microbiology, Government College University Faisalabad, Pakistan
[5]University Institute of Medical Lab Technology, The University of Lahore, Pakistan
[6]Department of Microbiology and Molecular Genetics, The Women University Multan, Pakistan

***Corresponding author:** Safdar Ali, Muhammad Rizwan
[*]Faculty of Science and Technology, Department of Molecular Biology, Virtual University of Pakistan, Email: dc210400273sal@vu.edu.pk
[*]Department of Microbiology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan, Email: muhammadrizwan_7862019@hotmail.com

Submitted: 15 December 2023; Accepted: 16 January 2024; Published: 18 March 2024

Abstract

Introduction: Typhoid fever is caused by *Salmonella typhi*, which is known for being resistant to several antibiotics. It is one of the leading causes of death and infections in developing nations, and also a frequent cause of illnesses in Pakistan. The major cause of this infection is to face a significant challenge in dealing with higher-level quinolone-resistant *S. typhi* strains. To combat this challenge a study was designed to determine the presence of the *qnr* genes among *S. typhi* isolated from stool of patient samples.

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, *S. typhi* was isolated, antibiotic susceptibility testing was carried out, and the bacterial genome was extracted by boiling method. PCR for detection of *qnr* genes including *qnr A, qnrB* and *qnrS* was done by specific primers, then PCR products were run using gel electrophoresis and visualized by gel documentation system.

Results: Out of 150 isolates, 13 (8.7%) 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 (92.3%), amoxicillin/clavulanic acid (84.6%), ceftriaxone/sulbactam

(84.6%), ciprofloxacin (84.6%), gentamicin (76.9%), levofloxacin (46.2%) and ofloxacin (46.2%).The most common antibiotic resistant phenotype was AUG- CTX-IMP-OFX-CN-NA-CXM-CRO-CIP-LBC at 30.4%. Multiple antibiotic resistance (MAR) was observed in 100% (13/13) of the isolates with the common MAR indices being 1.0 (30.8%), 0.7 (23.5%), 0.8 (23.1%) and 0.9 (15.4%). The only positive *PMQR* genes were *qnrS* and *aac(6')-Ib-cr* with percentage occurrence of 50.0% respectively.

Conclusion: The *S. typhi* isolates showed lower resistance to ofloxacin, levofloxacin, and gentamicin, and all isolates were MAR, with resistance to 10 antibiotics being the most predominant. In addition, *qnr*S resistance gene was the most common gene expressed.

Keywords: Salmonella, Typhoid fever, Gene, *qnrS*

Introduction

Quinolones are an important class of antibacterial drugs [1]. Initially, nalidixic acid, ca member of the class of drugs, had limited clinical use. But with chemical modifications, especially the addition of fluorine, fluoroquinolones was birthed. Fluoroquinolones (such as levofloxacin, ciprofloxacin) are more potent, have a broader spectrum of activity, and a lower frequency of resistance as recorded in several studies [2]. Fluoroquinolones have been used to treat many infections [3], including *Salmonella* infections, which can be dangerous, and even life-threatening [4]. The introduction of quinolones into human medicine has led to the emergence and increase of resistance to this class of antibiotics [5, 3].

Fluoroquinolones, in their mechanism of action, target 2 essential bacterial enzymes, DNA gyrase and topoisomerase IV. In *Enterobacteriaceae* including *Salmonella*, quinolone resistance is known to develop from the accumulation of chromosomal mutations in the quinolone resistance-determining region (*QRDR*) of the target enzyme genes (or targeted genes), primarily *gyrA* and *parC* [6, 3]. Since the late 1990s, 3 interesting types of plasmid-mediated quinolone resistance (*PMQR*) mechanisms have been identified: *qnr* genes, which protect target enzymes; *aac(6')-Ib-cr* gene, which is known to help in mediation and acetylation of certain quinolones; and *oqxAB* and *qepA* genes, which produce mobile efflux pumps [7, 8]. A *PMQR* gene alone usually offers decreased susceptibility to fluoroquinolones and has less effect on nalidixic acid susceptibility [2, 8].

The implications of *PMQR* in the treatment of infections and the dissemination of antimicrobial resistance are numerous and important. Plasmids harboring quinolone resistance genes can be horizontally transferred to other bacteria, thus spreading resistance. The aim of this study was to determine the prevalence of *S. typhi* and expression of *qnr* genes (*qnrA,qnrB,* and *qnrS*) in *S. typhi* isolated from the stool of patients in Bahawalpur Victoria Hospital (BVH), Pakistan.

Material and Methods

Media

Bacteriological media that were used in this study include: Salmonella-Shigella (S.S) 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); Peptone water (PW) all were obtained from Oxoid Ltd (U.K.).

Antibiotic Discs

The antibiotics discs used in this study and their potency are as follows: Amoxicillin/Clavulanate (AMC: 30 µg), Cefotaxime (CTX: 25 µg), Imipenem/Cilastatin (IMP: 10 µg), Ofloxacin (OFX: 5 µg), Gentamicin (CN: 10 µg), Nalidixic acid (NA: 30 µg), Cefuroxime (CXM: 30 µg), Ceftriaxone/Sulbactam (CRO: 30 µg), Ciprofloxacin (CIP: 5 µg), Levofloxacin (LBC: 5 µg). All the discs were sourced from Oxoid Ltd. UK.

Chemicals and Reagents

The chemicals and reagents that were used in this study include: Acridine orange, Carbol fuschin, Crystal violet, Ethanol, Xylene solution, Creatinine, Pottasium hydroxide and Kovac's reagents, obtained from BDH Chemical Ltd, England; Ethydium bromide, Iodine solution, EDTA and Glycerol obtained from Sigma Chemical Ltd, England; and Agarose gel, from Schwarz/ Mann Biotech.

Primers and their Amplicon Sizes

Primers were purchased from Inqaba Biotech (South Africa). The primers, sequences and amplicon sizes are as shown in Table 1.

Table 1. Primer Sequence and amplicon of target genes for *Salmonella typhi*

Target gene	Primer Sequence	Annealing Temp.(°C)	Amplicon size (bp)	Reference
qnrA	5'-CCGCTTTTATCAGTGTGACT-5' 3'-ACTCTATGCCAAAGCAGTTG -3'	55	188	[9]
qnrB	5'-GATCGTGAAAGCCAGAAAGG-5' 3'-ACGATGCCTGGTAGTTGTCC -3'	54	469	[9]
qnrC	5'-GGGTTGTACATTTATTGAATCG-5' 3'-CACCTACCCATTTATTTTCA -3'	54	308	[9]
qnrD	5'-CGAGATCAATTTACGGGGAATA-5' 3'-AACAAGCTGAAGCGCCTG - 3'	57	582	[9]
QnrS	5'-ACGACATTCGTCAACTGCAA-5' 3'-TAAATTGGCACCCTGTAGGC- 3'	55	417	[9]
aac(6')-Ib	5'-TTGCGATGCTCTATGAGTGGCTA-5' 3'-CTCGAATGCCTGGCGTGTTT- 3'	57	482	[9]
Class1Integron	5'-TCCACGCATCGTCAGGC-5' 3'-CCTCCCGCACGATGATC-3'	55	280	[9]

Place of Study

The study was carried out at Molecular department, Virtual University of Pakistan and department of Microbiology, Bahawalpur Victoria Hospital (BVH), Pakistan.

Sample Collection

A total of 150 stool samples of patients with suspected cases of typhoid fever were collected using sterile container and transported using ice pack to the department of Microbiology, Bahawalpur Victoria hospital (BVH) for culture and sensitivity and transport to the department of molecular biology, Virtual University of Pakistan for molecular analysis.

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 manufacturer's instruction.

Antimicrobial Susceptibility Testing

The antibiotic susceptibility test of the isolates was carried out as earlier described by Clinical and Laboratory Standards Institute [11]. Briefly, the 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) $BaCl_2.2H_2O$ was added into 99.5 ml of 1% (w/v) H_2SO_4 [11]. A sterile swab stick was soaked in the standardized bacteria suspension and streaked on Mueller-Hinton agar plates and the antibiotics disc were aseptically placed at the centre of the plates and allowed to stand for 1 h for diffusion. The plates were incubated at 37°C for 24 h. The diameter zone of inhibition in millimeter was measured and the

result of the susceptibility was interpreted in accordance with the susceptibility break point earlier described by Clinical and Laboratory Standards Institute [11].

Determination of Multiple Antibiotic Resistance (MAR) Index

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

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

Molecular detection of Quinolone resistance genes

DNA Extraction

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

Amplification of Target Genes

The DNA amplification of target plasmid–mediated Quinolone resistant genes in ciprofloxacin resistant *S. typhi* isolates were 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 $MgCl_2$, 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 94ºC for 5 min followed by 32 cycles of amplification at 94ºC for 45 sec each, annealing at 53ºC for 45 sec, with final extension at 72ºC for 5 min [14].

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

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].

Results

Isolation and Identification of Salmonella typhi

The organism which grew with Central Black Colonies on Salmonella-Shigella (SSA) Agar shown in (Figure 1), 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*.

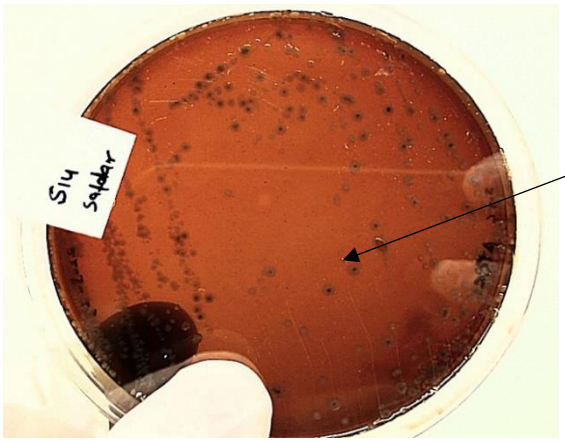

Figure 1. Central Black color colones on S.S Agar due to the H_2S Production indication of *Salmonella typhi.*

Gram stain for the morphology of Salmonella typhi

Microscopic examination of the stained smear reveals, the gram-negative rod-shaped bacterium, typically around 2-3 micrometers in length and 0.5 micrometers in diameter shown in (Figure 2) on a 100x oil immersion lens.

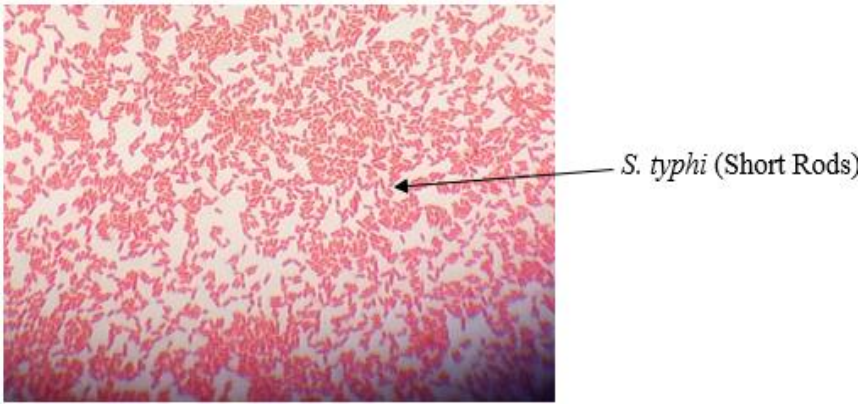

Figure 2. Stained slide under microscopy shows gram-negative rod-shaped *Salmonella typhi.*

Occurrence of Salmonella typhi

The isolation rate for *S. typhi* was 8.7 % (13/150). In relation to age of patients, the occurrence of *S. typhi* was highest at age 21-30yrs (16.7%), but lowest in age 41-50 (5.7%) as shown in Table 2.

Table 2. Occurrence of *Salmonella typhi* from stool of patients with suspected typhoid fever in Bahawalpur Victoria Hospital (BVH), Pakistan in relation to age

Age	**No of Samples**	**Number (%) *Salmonella typhi***
≤ 10	10	1(10.0)
11- 20	20	2(10.0)
21- 30	30	5(16.7)
31- 40	40	2(5.0)
41- 50	35	2(5.7)
> 50	15	1(6.7)
Total	150	13 (8.7)

Antimicrobial Resistance Profile

The Antibiotic Resistance of *S. typhi* isolates from of patients with suspected typhoid fever in Bahawalpur Victoria Hospital (BVH), Pakistan, is as given in Table 3. The isolates from BVH were

more resistant to Cefotaxime (100.0%), imipenem (100.0%), and ceftriaxone/Sulbactam (100.0%), but were less resistance to Levofloxacin (46.2%), and ofloxacin (46.2%).

Table 3. Antimicrobial resistance profile of *Salmonella typhi* from stool of patients with suspected typhoid fever in Bahawalpur Victoria Hospital (BVH), Pakistan

Antibiotics	**Disc Content (µg)**	**No. (%) resistance in *S. typhi* (n=13)**
Amoxicillin/Clavulanate (AMC)	30	11(84.6)
Cefotaxime (CTX)	25	13 (100.0)
Imipenem/Cilastatin (IMP)	10	13(100.0
Ofloxacin (OFX)	5	6(46.2)
Gentamicin (CN)	10	10(76.9)
Nalidixic acid (NA)	30	12(92.3)
Cefuroxime (CXM)	30	11(84.6)
Ceftriaxone/Sulbactam (CRO)	30	13(100.0)
Ciprofloxacin (CIP)	5	11(84.6)
Levofloxacin (LBC)	5	6(46.2)

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 13 isolates (100.0%). This suggests the possibility that most of the isolates originated from an environment where abuse of antibiotics was regular [12]. The most common indices were 1.0 (30.8%), 0.8 (23.1%), and 0.9 (15.4%).

Molecular Detection of Plasmid Mediated Quinolone resistant genes

The plasmid mediated quinolone resistant genes detected in quinolone resistant isolates is as shown in (Table 4). The only positive *PMQR* genes were *qnrS* and *aac(6′)-Ib-cr* with percentage occurrence of 50.0% respectively in (Table 5)**.**

Table 4. Multiple Antibiotics Resistance (MAR) Index of *Salmonella typhi* isolates from stool of patients from Bahawalpur Victoria Hospital (BVH), Pakistan.

No. of Antibiotic Resistance (a)	**No of Antibiotics Tested (b)**	**MAR Index (a/b)**	**No (%) of MAR Isolates (n =17)**
10	10	1.0	4(30.8)
9	10	0.9	2(15.4)
8	10	0.8	3(23.1)
7	10	0.7	1(7.7)
6	10	0.6	1(7.7)
5	10	0.5	1(7.7)
4	10	0.4	0(0.0)

Table 5. Molecular Detection of plasmid mediated Quinolone Resistance genes in Quinolone resistant *Salmonella typhi* Bahawalpur Victoria Hospital (BVH), Pakistan.

Quinolone resistance Genes	**No. (%) of *S. typhi*) n=6**
QnrA	1(16.7)
QnrC	1 (16.7)
QnrD	1 (16.7)
QnrS	2 (3.34)
aac(6′)-Ib-cr	1 (16.7)
qnrA + qnrC	2 (3.34)
qnrC + qnrD	2 (3.34)
qnrD + qnrS	3 (50.0)
qnrS + aac(6′)-Ib-cr	3 (50.0
qnrA + qnrC + qnrD +qnrS + aac(6′)-Ib-cr	6 (100.0)

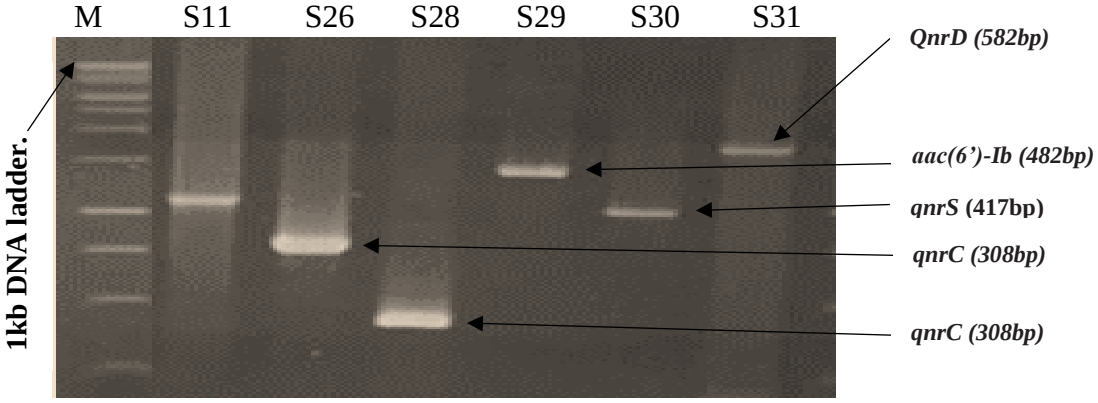

Figure 3: Agarose gel electrophoresis of the amplified quinolones resistance *genes* of *Salmonella typhi*. Lane S11 and Lane S30 represent the expression of the *qnrS (417bp)* gene; Lane S26 represent the expression of the *qnrC* (308bp) gene; Lane S28 represent the expression of the *qnrA (188bp)* gene; Lane S29 represent the expression of the *aac(6')-Ib (482bp)* gene; Lane S30 represent the expression of the *qnrS (417bp)* gene and Lane S31 represent the expression of the *QnrD (582bp)* gene; while Lane M represents 1kb DNA molecular ladder.

Discussion

A study by Jubair *et al* in Iraq [16], stated a low prevalence of the *S. typhi* strain in Iraq (13%). This isn't consistent with another study by Rahman *et al* [17] where other Middle Eastern countries had an 80.0% of *S. typhi* -infected patients, suggesting that most *S. typhi* infections originate from the Asian continent, while the remainder is primarily from Pakistan, Africa and Latin America [17].

This study used 150 stool samples collected from patients with suspected typhoid fever to isolate and diagnose *S. typhi*. In the current study, only 13(8.7%) positive samples identified as *S. typhi* based on colony morphology on culture medium, biochemical testing amongst others, This is lower than reports from a study of hospitals in Najaf, Iraq, where a higher rate of culture-positive typhoid fever isolates was reported in 2017 and 2020 [18, 19].

The occurrence of *S. typhi* from stool of patients in relationship to their age was higher at age 21-30 years, and this is in agreement with a study which reported high occurrence of *S. typhi* in patients of age > 10-41 years [17] and 21-31 years [20].

The high resistance of the isolates to antibiotics such as cefotaxime, imipenem, and ceftriaxone/Sulbactam as observed in this study was not surprising and may be due to abuse of the antibiotics. The high resistance of the isolates to imipenem, cefotaxime and ceftriaxone/Sulbactam (all 100.0%) was similar to findings from a study in Abuja by Fasema *et al* [21]. Furthermore, the low resistance of the isolates to antibiotics including Ofloxacin (46.2%), and levofloxacin (46.2%) is higher than findings by Dong *et al* [22], but lower than reports from a study in Iraq and Iran [23, 24]. The low resistance of these isolates to antibiotics makes a case for them to be presented as the foremost option for treatment of infections caused by *S. typhi* in this region.

Quinolones were the best option for antibiotics when classical MDR *S. typhi* first appeared, However, *S. typhi* isolates that are resistant to quinolones have emerged due to the extensive use of quinolones to treat typhoid fever [25]. This is no surprise as the Multiple antibiotic resistance (MAR), which is the resistance of microorganisms to at least two (2) antibiotics was observed in all 13 isolates (100.0%). The three most common *PMQR* genes (*qnrA, qnrB, and qnrs*) that have led to the persistent spread of quinolone-resistant *S. typhi* were chosen in this study. However, the only positive *PMQR* genes were *qnrS* and *aac(6')-Ib-cr* with percentage occurrence of 50.0% respectively**.** A study on *qnr* genes in *S. typhi* isolates from South Korea found that *qnrB* was the most common *qnr* gene [26]. In India, the *qnrB* gene was amplified in 70.0% of *S. typhi* strains isolated [27]. In Iran, *qnrA*

was found in 30.4% of the *S. typhi* isolates, *qnrS* was detected in 56.5%, while *qnrB* was detected in 1.1% [20].

Conclusion

This study showed that *S. typhi* is a cause of infection among typhoid patients, ofloxacin, and levofloxacin are fully effective against *S. typhi* clinical isolates from typhoid fever patients. All isolates considered were multiple drug resistant, and this is significant. The *qnrS* gene was present in some isolates. This gene may contribute to the quinolone resistance of *S. typhi*. The appearance of a *PMQR* gene in *S. typhi* means it is necessary for there to be a limit to the use of quinolones to reduce the spread of resistant strains.

Consent

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images. 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 start of the research and is available for reviews.

References

1. World Health Organization. Critically important antimicrobials for human medicine, 5th Revision 2016. 2017.http://www.who.int/foodsafety/publications/antimicrobials-fifth/en/.
2. Hooper DC, Jacoby GA. Mechanisms of drug resistance: quinolone resistance. *Annals of the New York academy of sciences*. 2015 Sep;1354(1):12-31.
3. Yanat B, Rodríguez-Martínez JM, Touati A. Plasmid-mediated quinolone resistance in Enterobacteriaceae: a systematic review with a focus on Mediterranean countries. European *Journal of Clinical Microbiology & Infectious Diseases*. 2017 Mar;36:421-35.
4. Crump JA, Barrett TJ, Nelson JT, Angulo FJ. Reevaluating fluoroquinolone breakpoints for *Salmonella enterica* serotype Typhi and for non-Typhi salmonellae. *Clinical Infectious Diseases*. 2003 Jul 1;37(1):75-81.
5. Van TT, Nguyen HN, Smooker PM, Coloe PJ. The antibiotic resistance characteristics of non-typhoidal *Salmonella enterica* isolated from food-producing animals, retail meat and humans in South East Asia. *International Journal of Food Microbiology*. 2012 Mar 15;154(3):98-106.
6. Cavaco LM, Aarestrup FM. Evaluation of quinolones for use in detection of determinants of acquired quinolone resistance, including the new transmissible resistance mechanisms qnrA, qnrB, qnrS, and aac (6′) Ib-cr, in *Escherichia coli* and *Salmonella enterica* and determinations of wild-type distributions. *Journal of Clinical Microbiology*. 2009 Sep;47(9):2751-8.
7. Jacoby GA, Strahilevitz J, Hooper DC. Plasmid-mediated quinolone resistance. Plasmids: *Biology and Impact in Biotechnology and Discovery*. 2015 May 30:475-503.
8. Rodríguez-Martínez JM, Machuca J, Cano ME, Calvo J, Martínez-Martínez L, & Pascual A(2016).
9. Plasmid-mediated quinolone resistance: Two decades on. *Drug Resistance Updates*, 29, 13–29. 10.1016/j.drup.2016.09.001. [PubMed: 27912841]
10. Herrera-Sánchez MP, Castro-Vargas RE, Fandiño-de-Rubio LC, Rodríguez-Hernández R,Rondón-Barragán IS. Molecular identification of fluoroquinolone resistance in *Salmonella* spp. Isolated from broiler farms and human samples obtained from two regions in Colombia. *Veterinary World*.2021 Jul;14 (7):1767.
11. Cheesbrough M. District laboratory practice in tropical countries: Cambridge university press; 2006.

12. Ali, S., Jalees, M. M., Ashraf, W., Sadia, M., Anwar, R., Zafar, & Mannan, A. (2024). Emergence Of Extensive Drug Resistance Typhoid In Hospitalized Covid-19 Patients In South Punjab, Pakistan. *Journal of Population Therapeutics and Clinical Pharmacology*, *31*(2), 753-763.
13. Krumperman PH. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Applied and environmental microbiology*. 1983 Jul; 46(1):165-70.
14. Abimiku RH, Ngwai YB, Nkene IH, Bassey BE, Tsaku PA, Ibrahim T, Tama SC, Ishaleku D, Pennap GR. Molecular Diversity and extended spectrum beta-lactamase resistance of diarrheagenic *Escherichia coli* from patients attending selected health care facilities in Nasarawa state, Nigeria. *International Journal of Pathogen Research*. 2019 Sep 2;3(1):1-8.
15. Jang S, Kelley KW, Johnson RW. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proceedings of the National Academy of Sciences. 2008 May 27;105(21):7534-9.
16. Aljanaby AA, Medhat AR. Research article prevalence of some antimicrobials resistance associated-genes in *Salmonella typhi* isolated from patients infected with typhoid fever. *J. Biol. Sci*. 2017;17(4):171-84.
17. Jubair HH, Al-Buhamrah NA, Al-Fatlawi SG. Molecular characterization of quinolones resistant *Salmonella typhi* isolates from patients infected with Typhoid fever in Al-Najaf province, Iraq. *Journal of Population Therapeutics and Clinical Pharmacology*. 2023 Mar 13;30(3):11-9.
18. Rahman BA, Wasfy MO, Maksoud MA, Hanna N, Dueger E, House B. Multi-drug resistance and reduced susceptibility to ciprofloxacin among *Salmonella enterica* serovar Typhi isolates from the Middle East and Central Asia. *New microbes new Infect*. 2014;2(4):88–92.
19. Aljanaby AAJ, Medhat AR. Research article prevalence of some antimicrobials resistance associated-genes in *Salmonella typhi* isolated from patients infected with typhoid fever. *J Biol Sci.*2017;17(4):171–84.
20. AL-Fatlawy HNK, AL-Hadrawi HAN. Molecular Profiling of Class I Integron Gene in MDR *Salmonella Typhi* Isolates. *J Pure Appl Microbiol*. 2020;14:1825–33.
21. Njum AA, Hassan RN, Alwan JA. Identification of Antibiotic-Resistant Genes in *Salmonella Typhi* Isolated From Typhoid Patient in Samawa City. *Iraqi J Sci*. 2019;60(5):980–4.
22. Fasema R, Bassey BE, Ngwai YB, Nkene IH, Abimiku RH, Parom SK, Yahaya I. Plasmid-mediated Quinolone Resistance Genes in *Salmonella typhi* from Patients Attending Selected General Hospitals in Abuja Municipal, Nigeria. Asian Journal of Biochemistry, *Genetics and Molecular Biology*. 2020 Jul 6;4(2):45-59.
23. Dong N, Li Y, Zhao J, Ma H, Wang J, Liang B, et al. The phenotypic and molecular characteristics, of antimicrobial resistance of *Salmonella enterica* subsp. enterica serovar Typhimurium in Henan Province, China. *BMC Infect Dis*. 2020;20(1):1–11.
24. Malehmir S, Ranjbar R, Harzandi N. The molecular study of antibiotic resistance to quinolones in *Salmonella enterica* strains isolated in Tehran, Iran. *Open Microbiol J*. 2017;11:189.
25. Salman HA, Abdulmohsen AM, Falih MN, Romi ZM. serovar Typhi isolated from Iraqi subjects. 2021;14:1922–8.
26. Tadesse G, Tessema TS, Beyene G, Aseffa A. Molecular epidemiology of fluoroquinolone resistant *Salmonella* in Africa: A systematic review and meta-analysis. PLoS *One.* 2018;13 (2):e0192575.
27. Kim H Bin, Park CH, Kim CJ, Kim E-C, Jacoby GA, Hooper DC. Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. *Antimicrob Agents Chemother*. 2009;53 (2):639–45.
28. Geetha VK, Yugendran T, Srinivasan R, Harish BN. Plasmid-mediated quinolone resistance in typhoidal *Salmonellae:* a preliminary report from South India. *Indian J Med Microbiol*. 2014;32(1):31–4.