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Molecular Characterization of Quinolone Resistance Determinants in Non-Typhoidal *Salmonella* Strains Isolated in Tehran, Iran

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Abstract

Background: Quinolone resistant *Salmonella* serotypes have been reported in recent years and have become increasingly widespread worldwide.
Objectives: We evaluated the molecular mechanism of quinolone resistance in non-typhoidal *Salmonella* strains isolated from clinical samples in Tehran, Iran.
Methods: The present study included the *Salmonella* isolates originated from hospitalized individuals and outpatients in Tehran, Iran. Serotyping of nalidixic acid-resistant *Salmonella* isolates was done by slide agglutination method. Then, the quinolone resistance-determining region (QRDR) of topoisomerase gene *gyrA* and the plasmid-mediated quinolone resistance (PMQR) determinants were detected using the polymerase chain reaction (PCR) method. Restriction fragment length polymorphism (RFLP) analysis was also employed to determine the possible mutation in the *gyrA* gene of those strains. Mutant strains were detected by enzymatic digestion, and their PCR products were sequenced immediately.
Results: Amongst 141 isolates, 60% showed nalidixic acid resistance, whereas none of them were ciprofloxacin-resistant. The commonly prevalent serotypes were S. *Enteritidis* and *S. Infantis*. Of 85 nalidixic acid-resistant strains, 17 (20%) isolates harbored the *qnrS* gene. However, PCR analysis of the quinolone-resistant strains did not detect *qnrA* and *qnrB* genes. PCR-RFLP and sequencing analysis of the QRDRs of the *gyrA* gene indicated that 16 (18.8%) isolates had mutant patterns, and the most common point mutation was serine to phenylalanine at position 83.
Conclusions: Our results demonstrated that point mutations in *gyrA* and the existence of plasmid-mediated gene *qnrS* were important mechanisms of quinolone resistance in non-typhoidal *Salmonella* strains isolated from human origin. Other alternative mechanisms of resistance, such as alterations in the expression of efflux pumps, should be studied to provide greater insight into the molecular mechanism of quinolone-resistant non-typhoidal *Salmonella* isolates.

Keywords: *Salmonella*, Nalidixic Acid, Serotype, Quinolones, Iran

1. Background

Salmonella is one of the most important foodborne pathogens responsible for an approximately three billion cases of diarrheal diseases each year (1, 2). For non-typhoidal salmonellosis, the mean infective dose to produce symptomatic disease in healthy adults is 10^5 - 10^8. A smaller inoculum, on the other hand, can induce infections in newborns and people with specific underlying conditions like human immunodeficiency virus (HIV) infection, cancer, or other immune suppressing conditions (3, 4).

Water and animal-derived foods, such as meat, poultry, eggs, and dairy products, as well as raw fruits and vegetables, can cause non-typhoidal *Salmonella* infections (5, 6). In contrast to the invasive salmonellosis that requires prompt antibiotic therapy, antibiotics are not usually crucial for the treatment of *Salmonella* gastroenteritis. Trimethoprim/sulfamethoxazole (SXT), fluoroquinolones, and oxyimino-cephalosporins are commonly recommended as therapeutic options for patients with severe infections (7). Quinolone-resistant *Salmonella* isolates in people have been reported often in recent years and have become increasingly widespread worldwide (8, 9). Misappropriation of prescribed antibiotics and horizontal gene transfer have been responsible for the increase of

Salmonella resistant to conventional antimicrobials (such as quinolones) in Iran (10, 11).

The main chromosomal point mutations occur in topoisomerase IV (*parC* and *parE*) and DNA *gyrA*se (*gyrA* and *gyrB*) encoding genes (10, 12). Meanwhile, quinolone resistance has been shown to be caused by changes in efflux pump production and DNA gyrAse protection by the qnr protein, which is created from plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*, and *aac(6′)Ib-cr*) on a conjugative plasmid or transposon (8, 13). Quinolone resistance in foodborne *Salmonella* could be linked to the occurrence of foodborne outbreaks and the subsequent problem of clinical treatment. Thus, understanding the antibiotic resistance molecular mechanisms, especially quinolones, in *Salmonella* isolates can be helpful.

2. Objectives

The present study aimed to investigate the occurrence of quinolone resistance among non-typhoidal *Salmonella* strains isolated from patients in Tehran, Iran, and to elucidate the mechanisms behind it.

3. Methods

The present study involved *Salmonella* isolates originated from hospitalized individuals and outpatients in Tehran, Iran, based on laboratory confirmation and clinical presentations as described previously (7). A total of 141 *Salmonella* clinical strains were isolated, among which 113 (80%) strains were collected from stools, and 28 (20%) strains from blood, wound, urine, or other biological fluids. Only one isolate per patient was included in this study. Nalidixic acid-resistant *Salmonella* isolates were serotyped by slide agglutination method with anti-O and H antisera (Staten Serum Institute, Copenhagen, Denmark). Until use, the validated isolates were kept at -70°C in tryptic soy broth (TSB, Merck KGaA, Darmstadt, Germany) with 25% (v/v) glycerol (14). All ethical issues, including the subjects' health issues, dignity, integrity, right to self-determination, privacy, and confidentiality of personal information were considered.

According to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), antimicrobial susceptibility to nalidixic acid (30 μg) and ciprfloxacin (5 μg) was performed on Mueller-Hinton agar (Oxoid Ltd.) using the Kirby-Bauer disk diffusion method (15). As reported previously, the method for DNA extraction was boiling. To amplify the quinolone resistance-determining region (QRDR) of topoisomerase genes *gyrA*, *qnrS*, *qnrA*, and *qnrB* among the nalidixic acid-resistant strains, the primer sets listed in Table 1 were used. Polymerase chain reaction (PCR) was performed in a volume of 25 μL using a thermal cycler (Eppendorf, Hamburg, Germany) for 30 cycles, as previously described. The cycling conditions were as follows: an initial denaturation at 94°C for 10 minutes followed by denaturation at 94°C for 1 minute, primer annealing at 52 - 60°C for 1 minute, primer extension at 72°C for 1 minute, and the final extension at 72°C for 7 minutes. The PCR products were separated by electrophoresis at 100V for 2 hours on 1.5% (w/v) agarose gels, and visualized using an ultraviolet (UV) transilluminator (Tanon, Shanghai, China).

Restriction fragment length polymorphism (RFLP) was performed to determine the likely mutation in the *gyrA* gene among nalidixic acid-resistant strains. Briefly, 12 μL of PCR-amplified fragment was digested with 1 μL of *HinfI* (MBI Fermentas) in 3 μL digestion buffer in a final volume of 20 μL, and incubated at 37°C. After overnight incubation at 37°C, restriction fragments were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and examined by UV transillumination before being photographed. To identify mutant isolates, PCR products were sequenced immediately (Pishgam co., Tehran, Iran). The DNA sequences of the mutant strains were deposited into the GenBank database (ncbi.nlm.nih.gov) under accession numbers KF975705, KF975707, KF975709, KF975710, and KF975711. As shown in Figure 1, nucleotide sequences obtained from sequencing analysis were translated into amino acid sequences and aligned by the Multiple Alignment using Fast Fourier Transform (MAFFT) program (mafft.cbrc.jp).

4. Results

Amongst the 141 *Salmonella* isolates, 60% were nalidixic acid-resistant strains. However, none of them were resistant to ciprofloxacin or showed reduced susceptibility to it. Also, a total of seven different serotypes were identified. The commonly prevalent serotypes were S. *Enteritidis* and *S. Infantis* (Table 2). According to our results, 17 (20%) of the 85 nalidixic acid-resistant strains carried the *qnrS* gene, all of which belonged to the *Enteritidis* serotype. Furthermore, the *qnrA* and *qnrB* genes were not found in nalidixic acid-resistant strains.

Non-mutant and mutant PCR-amplified *gyrA* fragments have three and two *HinfI* restriction sites, respectively (Table 3). Restriction analysis of the PCR product revealed that 16 (18.8%) isolates showed mutant pattern. Sequencing of *gyrA* PCR products was used to confirm mutations associated with nalidixic acid resistance in 16 resistant organisms that displayed two bands in PCR-RFLP. Nucleotide sequencing showed that 16 isolates had mutations in *gyrA* in terms of change of the amino acid serine to

Table 1. PCR Primers Used in This Study

Gene	Primer	Primer Sequence (5'→3')	Amplicon Size (bp)	Annealing Temperature (°C)	Reference
gyrA	*DsgyrA*	F: GAC GGA TCT CCG TAT AAC	317	52	This study
		R: TCT GGA TTA TGC GAT CTC			
gyrA	*LsgyrA*	F: TGT CCG AGA TGG CCT GAA GC	347	60	(16)
		R: TAC CGT CAT AGT TAT CCA CG			
qnrA	D*sqnrA*	F: ATT TCT CAC GCC AGG ATT TG	516	55	(17)
		R: GAT CGG CAA AGG TTA GGT CA			
qnrB	*DsqnrB*	F: GTT GGC GAA AAA ATT GAC AGA A	500	52	(18)
		R: ACT CCG AAT TGG TCA GAT CG			
qnrS	*DsqnrS*	F: ACG ACA TTC GTC AAC TGC AA	417	55	(18)
		R: TTA ATT GGC ACC CTG TAG GC			

Table 2. Distribution of Serotypes Among Nalidixic Acid-Resistant *Salmonella* Isolates

Salmonella Serotype	Nalidixic Acid Resistant Isolates, No. (%)
Enteritidis	39 (45.88)
Infantis	35 (41.18)
Albany	4 (4.71)
Hadar	2 (2.35)
Munchen	2 (2.35)
Typhimurium	2 (2.35)
Haifa	1 (1.17)
Total	85 (100)

phenylalanine (Ser83→Phe, 7 isolates), serine to tyrosine (Ser83→Tyr, 5 isolates), aspartate to tyrosine (Asp87→Tyr, 3 isolates), and proline to leucine (Pro43→Leu, 1 isolate) (Figure 1). Amongst the seven isolates containing the substitution Ser83→Phe in *gyrA*, one isolate had multiple mutations (Ala51→Thr, Ser83→Phe, Val85→Leu, Ile89→Leu, Val90→Asp, Ala93→Ser, Gln94→His, Gly110→Arg, Ala117→Thr, and Ala118→Pro). Furthermore, two isolates harbored mutation at position 83 (Ser83→Phe) and carried *qnrS* gene (GenBank Accession Numbers: KF975711.1, KF975710.1, KF975709.1, KF975708.1, KF975707.1, KF975706.1, KF975705.1, KF975704.1, KC470542.1).

5. Discussion

Salmonella infections are common in many developing nations, including Iran. They rarely appear as a severe public health hazard in the industrialized countries. In previous investigations conducted in Iran, *S. enterica* serotype *Enteritidis* was found to be the most common serotype of *Salmonella* in people. Moreover, in a comprehensive study conducted in China, United States, and Taiwan, *S. enterica* serotype *Enteritidis* was the most prevalent strain serotype among human isolates (19-22). These results are in agreement with our results, which revealed that *S. enterica* serotype *Enteritidis* was the predominant serotype. However, other studies in Ghana, Armenia, and Georgia reported *S. enterica* serotype *Typhimurium* as the main serotype (14, 23). In humans, S. *Enteritidis* infections are most usually linked to contaminated chicken and its products, whereas *S. Typhimurium* infections are mostly linked to infected pig and bovine derivatives (2). Local agriculture and farming practices, food distribution and consumption patterns, as well as food consumer preferences and behaviors could play a role in *Salmonella* serotype ranking in a well-defined geographical area like Tehran, Iran.

Over the past decade, since these organisms have been linked to clinical failures of therapy and a considerable burden of hospitalization, the high incidence of antimicrobial-resistant *Salmonella* isolates has been a critical public health concern (24-26). Resistance to nalidixic acid, for example, is a concerning scenario because fluoroquinolones are the most often used antibiotics for the treatment of invasive salmonellosis in adults, and failure of therapy in individuals with nalidixic acid-resistant *Salmonella* infections has been observed (27). In Iran, resistance to nalidixic acid has been increased among nontyphoidal *S. enterica* isolates (27), as in our study, 60% of isolates were resistant to nalidixic acid. In a previous study in Iran, a high level of resistance to nalidixic acid among *Salmonella* strains has been reported (28). In this regard, a meta-analysis study investigated fluoroquinolone-resistant clinical strains in Iran. According to their results,

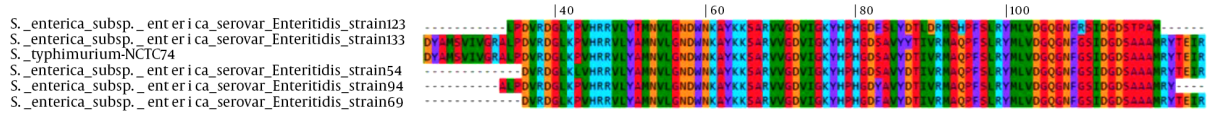

Figure 1. Alignment of the *gyrA* QRDR amino acid sequences of five *Salmonella enterica* subspecies *enterica* serotype *Enteritidis* with analogous sequence from reference strain of *Salmonella Typhimurium*_NCTC74.

Table 3. *HinfI* Restriction Pattern and the Length of Resulting Fragments

Gene and Primer	Amplicon Size (bp)	Position of Restriction Sites	Restriction Fragment Length (bp)	
			Non-mutant	Mutant
gyrA/ LsgyrA	347	108 and 207	108, 99, and 140	108 and 239 or 207 and 140
gyrA/ DsgyrA	317	33 and 132	33,99 and 185	33 and 284 or 132 and 185

the pooled prevalence of nalidixic acid-resistant isolates in Iran was 48.1%. Moreover, from 1983 to 2019, the nalidixic acid-resistance trend of *Salmonella* serotypes was increasing in Iran (11).

In contrast, our results regarding the resistance to nalidixic acid in human isolates of S. *Enteritidis* were higher than those of South East Asian countries, including Malaysia, Thailand, and Vietnam (27 - 38%) (29-31). In addition to nalidixic acid, no ciprfloxacin-resistant isolate was found in the present study. This finding is in line with the current situation in Tehran, where no ciprofloxacin-resistant isolates were detected among 174 *S. enterica* strains (32). However, a comprehensive analysis by Khademi et al. revealed that the pooled occurrence of ciprofloxacin-resistant *Salmonella* serotypes in clinical specimens was 2.9% in Iran (11). In *Salmonella* spp., point mutations at QRDR of DNA *gyrA*se are mainly the cause of quinolone resistance between amino acids 67 and 106, which can alter the binding site of the agents with DNA *gyrA*se (33). Single mutations in the *gyrA* gene have been discovered to be sufficient to provide substantial levels of resistance to quinolones like nalidixic acid in *Salmonella* spp. (34).

In the present study, genetic characterization of nalidixic acid resistance revealed that all mutant *S. enterica* isolates had point mutations at codons 83 or 87 of *GyrA*, except one isolate that had point mutation at position 43 (Pro43→Leu), which was not previously reported. Double substitutions at both positions 83 and 87 were not identified in this study. The most common point mutation of *gyrA* occurred at position 83 (Ser→Phe, 43.7%). In contrast to our finding, previous reports from Iran showed that the predominant substitution was at position 87 (Asp87→Asn) of *GyrA* (32, 35). In agreement with the current study, the highest prevalence of amino acid substitution in *GyrA* was Ser83→Phe (92.1%) among nalidixic acid-resistant *S. enterica* serotype *Typhi* strains, isolated from southern Vietnam (36). Indeed, these reports from different countries indicate that the presence of mutation at Ser83 may cause reduced susceptibility to ciprofloxacin, representing high level of nalidixic acid resistance (36).

Plasmid-borne resistance, such as PMQR in *S. enterica*, is a public health concern because it results in horizontal fluoroquinolone resistance transfer between strains (37). In this study, 20% of isolates harbored the *qnrS* gene. Moreover, PCR analysis of the quinolone-resistant strains did not detect *qnrA* or *qnrB* genes. These findings partly agree with previous studies conducted in Korea and United States (10, 38). There are few studies investigating the PMQR in non-typhoidal *Salmonella* isolates in Iran. However, a recent study by Saboohi et al. in Iran demonstrated that 25.8%, 1.17%, and 1.17% of *Salmonella* spp. harbored *qnrA*, *qnrB*, and *qnrS* genes, respectively (39). Contrary to our finding, Abbasi and Ghaznavi-Rad revealed that among the PMQR genes, *qnrS*, *qnrA*, and *qnrB* were positive in 60%, 40%, and 20% of the isolates, respectively (28).

5.1. Conclusions

This study provided an insight into the molecular mechanism of quinolone resistance in non-typhoidal *Salmonella* strains isolated from patients in Tehran, Iran. We cannot rule out the presence of mutations outside of the sequenced region, but our findings suggest that other possible mechanisms may play a role regarding the quinolone resistance in *Salmonella* isolates. These mechanisms could include *parE* mutations, changes in expression of eflux pumps, modifications of the outer membrane proteins or even novel mechanisms. Further studies are required to monitor the spread of non-typhoidal *Salmonella* involving QRDR and PMQR carriers and to determine other mechanisms of quinolone resistance.

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Footnotes

Authors' Contribution: Study concept and design: RR, CM and RH; Analysis and interpretation of data: AN and RH; Drafting of the manuscript: AN, MMb, and RH; Critical revision of the manuscript for important intellectual content: RR.

Conflict of Interests: The authors declare that they have no competing interests.

Data Reproducibility: The data presented in this study are openly available in one of the repositories or will be available on request from the corresponding author by this journal representative at any time during submission or after publication. Otherwise, all consequences of possible withdrawal or future retraction will be with the corresponding author.

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