



## Quinolone susceptibility and genetic characterization of *Salmonella enterica* subsp. *enterica* isolated from pet turtles

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Turtle-borne *Salmonella enterica* owns significance as a leading cause in human salmonellosis. The current study aimed to determine the quinolone susceptibility and the genetic characteristics of 21 strains of *S. enterica* subsp. *enterica* isolated from pet turtles. Susceptibility of four antimicrobials including nalidixic acid, ciprofloxacin, ofloxacin, and levofloxacin was examined in disk diffusion and MIC tests where the majority of the isolates were susceptible to all tested quinolones. In genetic characterization, none of the isolates were positive for *qnr* or *aac(6)-Ib* genes and no any target site mutations could be detected in *gyrA*, *gyrB*, and *parC* quinolone resistance determining regions (QRDR). In addition, neighbor-joining phylogenetic tree derived using *gyrA* gene sequences exhibited two distinct clads comprising; first, current study isolates, and second, quinolone-resistant isolates of human and animal origin. All results suggest that studied strains of *S. enterica* subsp. *enterica* isolated from pet turtles are susceptible to quinolones and genetically more conserved with regards to *gyrA* gene region.

**Keywords:** *Salmonella enterica* subsp. *enterica*, quinolone susceptibility, pet turtles, *qnr* genes, QRDR

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A well-adapted microbial pathogen, *Salmonella enterica* is a gram-negative, facultative anaerobic bacterium, which belongs to family Enterobacteriaceae. It was found to cause a variety of illnesses ranging from common food poisoning to severe typhoid fever [1]. Especially, salmonellosis is known as a main public health problem worldwide both in humans and domestic animals [2,3]. Human cases of salmonellosis were often associated with food [4], but frequently people got infected by handling infected animals and contaminated water and soil [5]. Children below the age of 5 years, elderly people and immunosuppressed patients were noted more susceptible to *Salmonella* infection than healthy individuals [6].

Quinolones are often used to treat invasive salmonellosis in humans and animals, but past studies encountered a significant number of clinical isolates

resistant or with a reduced susceptibility to these antimicrobials [7,8]. *Salmonella* strains isolated from humans and animals were reported to have nalidixic acid resistance and considerably reduced susceptibility to fluoroquinolones [9,10]. Quinolone resistance among *Salmonella* spp. is generally associated with mutations in the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*) that is possessed in the quinolone resistance-determining region (QRDR) [11]. However, the production of Qnr proteins by plasmid-mediated quinolone resistance (PMQR) genes was also documented as an another mechanism leading to quinolone-resistance [12]. These PMQR genes include; DNA gyrase protection from fluoroquinolone caused by a cluster of proteins encoded by *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS* and a variant of aminoglycoside acetyltransferase capable of acetylating several fluoroquinolone compounds,

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*aac(6')-Ib-cr* [13].

Chelonians, consisting of turtles, tortoises, and terrapins, are kept in captivity for commercial purposes, and mostly as pets. So far, turtle keeping trend is continuously increasing but, the turtle-associated medical knowledge still keeps slow growing. However, pet turtles are known to harbor a variety of zoonotic bacteria, especially, in their digestive tract [14,15]. Several previous studies have been conducted to assess the bacterial flora harbored by pet turtles and their environment [16-18]. Turtle-borne *Salmonella*, in particular, has been well-recognized as a major cause of salmonellosis in humans [19]. Due to the concerns about the excretion of pathogenic *Salmonella* spp. the sale of small carapace turtles was banned in the United States' domestic pet market in 1975 by the Food and Drug Administration [20]. In the meantime, plasmid mediated high-level gentamicin resistance was detected in *S. enterica* isolates from pet turtles in Louisiana, USA [21]. Both antimicrobial-resistant and susceptible *Salmonella* spp. has also been isolated from Russian tortoises and spur-thighed tortoises [22,23]. Another recent study recorded antibiotic-resistant *S. enterica* isolated from chelonians which were also harboring some virulence genes in their plasmids [24]. Besides, *S. enterica* could be isolated from several popular species of pet turtles and their environment indicating a potential risk of infection to turtle keepers [25].

Current study sought to characterize the *S. enterica* isolated from six commercially popular pet turtle species through the detection of quinolone susceptibility and screening the plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*) and chromosomal QRDR mutations in *gyrA*, *gyrB* and *parC* for a better understanding of a well-known turtle-borne pathogen.

## Materials and Methods

### Purchase of pet turtles

A total of thirty-five turtles comprising of 11 Chinese stripe-necked turtles (*Ocadia sinensis*), 8 yellow-bellied sliders (*Trachemys scripta scripta*), 6 river cooters (*Pseudemys concinna concinna*), 4 northern Chinese softshell turtles (*Pelodiscus maackii*), 3 western painted turtles (*Chrysemys picta belli*) and 3 common musk turtles (*Sternotherus odoratus*) were purchased from several pet shops and online markets in Korea. The purchased turtles had an average weight of  $15\pm 2$  g,

carapace diameter of  $40\pm 5$  mm, and were under 4 weeks of age. Turtles were raised under laboratory conditions following the general husbandry method [26].

### Isolation of *Salmonella* spp.

Fecal samples from turtles were enriched in tetrathionate broth (MBcell Ltd., Seoul, Korea) by incubation at 37°C for 24 h. Enriched samples were streaked onto the selective culture media, brilliant green agar (BGA, MB cell Ltd., Seoul, Korea) and xylose lysine desoxycholate agar (XLD, MB cell Ltd., Seoul, Korea) and incubated at 37°C for 24 hr. The isolates that showed the positive growth were presumptively identified as *Salmonella* spp.

### Bacterial identification using 16S rRNA gene amplification and sequencing

Genomic DNA was extracted from presumptively identified *Salmonella* spp. isolates by Chelex-100 extraction method and PCR for 16S rRNA was performed using universal primers 12F and 1492R. Amplicons were sequenced and tested for the similarity using Basic Local Alignment Search Tool (BLAST) algorithm of NCBI database so as to confirm the species status.

### Quinolone susceptibility testing

Twenty-one identified *S. enterica* isolates were selected for the study. Susceptibility pattern of the *S. enterica* isolates was investigated for nalidixic acid, ciprofloxacin, ofloxacin, and levofloxacin by disk diffusion test on Mueller Hinton agar (MBcell Ltd., Seoul, Korea). Minimum inhibitory concentrations (MIC) of nalidixic acid, ciprofloxacin and ofloxacin were detected by broth microdilution method. All susceptibility testing were conducted according to the recommendations of Performance Standards for Antimicrobial Susceptibility Testing; Clinical and Laboratory Standards Institute [27].

### Detection of quinolone resistance genes

The presence of *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib* genes were detected using primers and PCR conditions listed in Table 1. Each PCR mixture was 20  $\mu$ L total volume comprising of 10  $\mu$ L of Quick Taq<sup>®</sup> HS DyeMix (TOYOBO, Japan) 1  $\mu$ L of 10 pmol/ $\mu$ L each primer and 1  $\mu$ L of the template. PCR products were checked in 1.5% agarose gel. Amplicons of *aac(6')-Ib* gene were purified using Expin<sup>™</sup> PCR SV kit (GeneAll<sup>®</sup>, Korea) and were sent to Cosmogenetech Co. Ltd, Daejeon,

**Table 1.** Primers used to amplify QRDR and *qnr* genes and conditions of each reaction

Target	Primer	Nucleotide Sequence (5'-3')	Size (bp)	PCR conditions					Source of primer sequence
				Pre-denaturation	Denaturation	Annealing	Elongation	Final elongation	
<i>gyrA</i>	gyrA-F	CGTTGGTGACGTAATCGGTA	251	95°C, 5 min <sup>a</sup>	95°C, 1 min, 35x	55°C, 45 sec, 35x	72°C, 1 min, 35x	72°C, 7 min	[11]
	gyrA-R	CCGTACCGTCATAGTTATCC							
<i>gyrB</i>	gyrB-F	GCGCTGTCCGAACGTGTACCT	181	95°C, 5 min <sup>a</sup>	95°C, 1 min, 35x	55°C, 45 sec, 35x	72°C, 1 min, 35x	72°C, 7 min	[11]
	gyrB-R	TGATCAGCGTCGCCACTTCC							
<i>parC</i>	parC-F	CTATGCGATGTCAGAGCTGG	270	95°C, 3 min <sup>a</sup>	95°C, 1 min, 35x	55°C, 45 sec, 35x	72°C, 1 min, 35x	72°C, 7 min	[11]
	parC-R	TAACAGCAGCTCGGCGTATT							
<i>qnrA</i>	qnrA-F	ATTTCTCACGCCAGGATTTG	516	94°C, 5 min <sup>b</sup>	94°C, 45 s, 32x	53°C, 45 s, 32x	72°C, 1 min, 32x	72°C, 7 min	[36]
	qnrA-R	GATCGGCAAAGGTTAGGTCA							
<i>qnrB</i>	qnrB-F	GATCGGCAAAGGTTAGGTCA	469	94°C, 5 min <sup>b</sup>	94°C, 45 s, 32x	54°C, 45 s, 32x	72°C, 1 min, 32x	72°C, 7 min	[36]
	qnrB-R	ACGATGCCTGGTAGTTGTCC							
<i>qnrS</i>	qnrS-F	ACTGCAAGTTCATTGAACAG	431	95°C, 5 min <sup>b</sup>	95°C, 1 min, 32x	58°C, 1 min, 32x	72°C, 1 min, 32x	72°C, 7 min	[36]
	qnrS-R	GATCTAAACCGTCGAGTTTCG							
<i>aac(6)-Ib</i>	aac(6)-Ib-F	TTGCGATGCTCTATGAGTGGCTA	482	94°C, 5 min <sup>b</sup>	94°C, 45 s, 35x	55°C, 45 s, 35x	72°C, 45 s, 35x	72°C, 10 min	[39]
	aac(6)-Ib-R	CTCGAATGCCTGGCGTGTTC							

<sup>a</sup>PCR conditions were modified for this study<sup>b</sup>PCR conditions were adapted from the reference source

**Table 2.** Details of the *gyrA* gene sequences of *S. enterica* subsp. *enterica* downloaded from NCBI database for the phylogenetic analysis

NCBI accession number	Origin	Country of record
AJ919282.1	Quinolone-resistant animal isolate	UK
FJ222660.1	Ciprofloxacin-resistant human clinical isolate	Kuwait
GU330246.1	Quinolone-resistant chicken isolate	Korea
HQ176368.1	Human clinical isolate with reduced susceptibility to ciprofloxacin	India
HQ448882.1	Fluoroquinolone-resistant isolate from imported food	USA
KC121321.1	Nalidixic acid resistant isolate from fish	India
KP184391.1	Quinolone resistant human clinical isolate	China
KP290113.1	Quinolone-resistant poultry isolate	Egypt
EU512996.1 (out-group)	<i>Escherichia coli</i> ; quinolone resistant human isolate	Korea

Korea for sequencing. Acquired sequences were tested for the similarity in BLAST option in NCBI database in order to check the presence of *aac(6')-Ib-cr* gene variant.

#### Amplification of Quinolone Resistance Determining Region (QRDR) and detection of mutations

PCR for *gyrA* and *parC* regions were performed using primers and conditions described in Table 1. PCR was conducted in 50 µL volumes consisting of 20 µL of Quick Taq<sup>®</sup> HS DyeMix (TOYOBO, Japan), 2 µL of 10 pmol/µL each primer and 2 µL of the template. Amplicons were purified using Expin<sup>™</sup> PCR SV kit (GeneAll<sup>®</sup>, Korea) and sent to Cosmogenetech Co. Ltd., Daejeon, Korea for sequencing. Acquired sequences were subjected to detection of mutations by comparison with published NCBI reference sequences. Analyzing and comparison of QRDR sequences were performed using Mutation Surveyor V5.0.1 (Softgenetics LLC, USA) software.

#### Phylogenetic comparison of *gyrA* sequences with published sequences

The sequences acquired for *gyrA* gene region were analyzed and a neighbor-joining phylogenetic tree was derived with 1000 bootstrap replications. For the analysis, eight previously published *gyrA* sequences of *S. enterica* subsp. *enterica* clinical isolates were obtained from the GenBank database (see Table 2 for the accession numbers and details) and MEGA6 sequence analyzing software was used for aligning and construction of the phylogenetic tree.

## Results

#### Isolation and identification of *Salmonella* spp.

Twenty-one out of thirty-five isolates showed positive

growth on BGA and XLD agar. A subsequent BLAST search after 16S rRNA sequencing indicated a 99-100% match to *S. enterica* sequences available in GenBank which confirmed their identity as *S. enterica* subsp. *enterica*.

#### Quinolone susceptibility testing

The results of the quinolone susceptibility testing are shown in Table 3. In disk diffusion test, only three isolates showed intermediate resistance to nalidixic acid and two of them showed reduced susceptibility in MIC. All tested strains are susceptible to other three fluoroquinolones; ciprofloxacin, ofloxacin, and levofloxacin both in disk diffusion test and MIC.

#### Detection of quinolone resistance genes (*qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*) and QRDR mutations:

None of the isolates harbored *qnr* genes or *aac(6')-Ib*. Amplicon sequences of *gyrA*, *gyrB*, and *parC* did not show any specific point mutation in QRDR.

#### Phylogenetic comparison of *gyrA* sequences

Phylogenetic tree derived by analyzing and comparing the *gyrA* gene sequences obtained by current study and the published NCBI sequences is illustrated in Figure 1. Neighbor-joining phylogenetic tree indicated two distinct clads comprising first, current study isolates, second, quinolone-resistant isolates of human and animal origin.

## Discussion

It is reasonable to deem that *S. enterica* is a pathogen which is able to cause serious infections in animals and human. In the meantime, its intrinsic and acquired resistance to antimicrobials has also been well studied. Due to higher significance and implications counting

**Table 3.** Quinolone susceptibility pattern of *S. enterica* subsp. *enterica* isolated from pet turtles

Isolate*	Disk diffusion zone diameters (mm) <sup>a</sup>				MIC (µg/mL) <sup>b</sup>			
	NDX30	OFX5	CIP5	LEV5	NDX	CIP	OFX	LEV
YB1	24 (S)	31 (S)	35 (S)	33 (S)	8 (S)	0.015 (S)	0.06 (S)	0.030 (S)
YB2	22 (S)	32 (S)	36 (S)	33 (S)	4 (S)	0.030 (S)	0.06 (S)	0.030 (S)
YB3	22 (S)	30 (S)	35 (S)	32 (S)	4 (S)	0.030 (S)	0.06 (S)	0.030 (S)
YB4	18 (I)	30 (S)	32 (S)	32 (S)	16 (S)	0.030 (S)	0.06 (S)	0.030 (S)
RC1	18 (I)	31 (S)	34 (S)	32 (S)	8 (S)	0.030 (S)	0.06 (S)	0.030 (S)
RC2	18 (I)	30 (S)	32 (S)	33 (S)	16 (S)	0.030 (S)	0.06 (S)	0.030 (S)
RC3	22 (S)	32 (S)	36 (S)	34 (S)	4 (S)	0.015 (S)	0.06 (S)	0.015 (S)
CM1	21 (S)	31 (S)	34 (S)	32 (S)	4 (S)	0.015 (S)	0.06 (S)	0.030 (S)
CM2	23 (S)	33 (S)	37 (S)	35 (S)	4 (S)	0.015 (S)	0.03 (S)	0.015 (S)
CSS1	25 (S)	32 (S)	37 (S)	35 (S)	4 (S)	0.015 (S)	0.06 (S)	0.030 (S)
CSS2	23 (S)	31 (S)	36 (S)	33 (S)	8 (S)	0.015 (S)	0.06 (S)	0.030 (S)
WP1	24 (S)	31 (S)	34 (S)	33 (S)	8 (S)	0.030 (S)	0.06 (S)	0.030 (S)
CSN1	23 (S)	32 (S)	32 (S)	35 (S)	8 (S)	0.015 (S)	0.06 (S)	0.030 (S)
CSN2	24 (S)	32 (S)	34 (S)	33 (S)	4 (S)	0.015 (S)	0.03 (S)	0.015 (S)
CSN3	23 (S)	33 (S)	32 (S)	34 (S)	4 (S)	0.030 (S)	0.06 (S)	0.030 (S)
CSN4	24 (S)	30 (S)	33 (S)	34 (S)	4 (S)	0.015 (S)	0.06 (S)	0.030 (S)
CSN5	24 (S)	31 (S)	36 (S)	34 (S)	4 (S)	0.015 (S)	0.03 (S)	0.030 (S)
CSN6	22 (S)	32 (S)	37 (S)	33 (S)	8 (S)	0.030 (S)	0.06 (S)	0.015 (S)
CSN7	20 (S)	32 (S)	36 (S)	33 (S)	8 (S)	0.030 (S)	0.06 (S)	0.030 (S)
CSN8	19 (S)	33 (S)	39 (S)	36 (S)	8 (S)	0.015 (S)	0.03 (S)	0.015 (S)
CSN9	23 (S)	31 (S)	36 (S)	35 (S)	8 (S)	0.030 (S)	0.06 (S)	0.015 (S)

<sup>a</sup>Disk diffusion zone diameters (mm): NDX30=nalidixic acid (30 µg), OFX5=ofloxacin (5 µg), CIP5=ciprofloxacin (5 µg), LEV5=levofloxacin (5 µg); S=susceptible, I=intermediate, R=resistant were designated using breakpoints described by the Clinical Laboratory Standards Institute (CLSI-2014) [27].

<sup>b</sup>MIC (µg/mL): S=susceptible, I=intermediate, R=resistant were designated using breakpoints described by the Clinical Laboratory Standards Institute (CLSI-2014) [27].

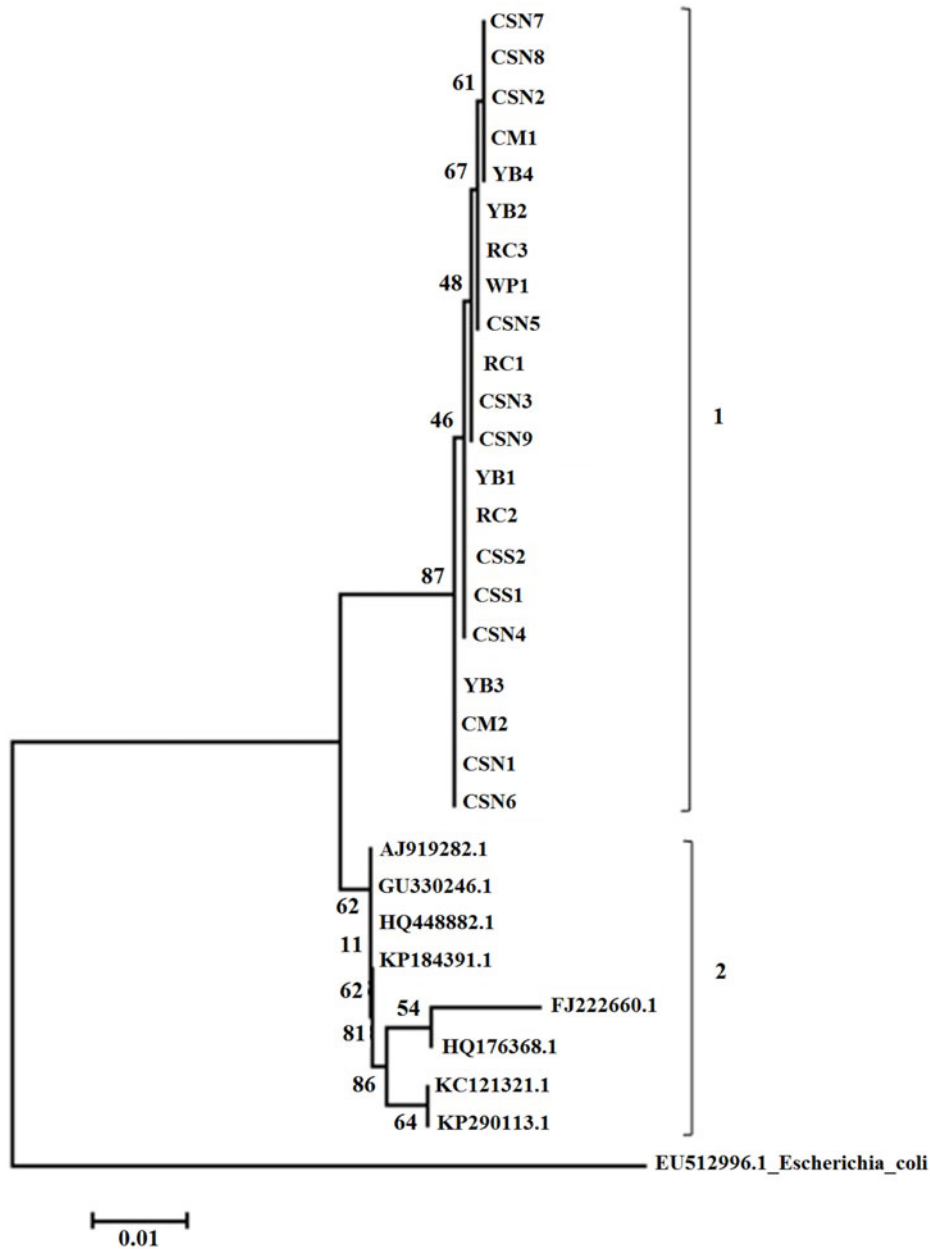
\*Isolate number was given according to the turtle species from which the strains were isolated: YB=yellow-bellied slider, RC=river cooter, CM=common musk turtle, CSS=Chinese softshell turtle, WP=western painted turtle, CSN=Chinese stripe-necked turtle.

current health concerns, studies associated with quinolone resistance, particularly genetics-based resistance, of *S. enterica* worth carrying out.

In this study, the majority of the isolates were susceptible to all tested quinolones except three isolates showing intermediate resistance to nalidixic acid. This outcome is in line with previous studies in which animal, especially reptile-borne *S. enterica* was examined. Nowakiewicz *et al.* reported *S. enterica* subsp. *enterica* strains isolated from Russian tortoises showing sensitivity to norfloxacin and other antibiotics [22]. *S. enterica* isolated from spur-thighed tortoises and tegu lizards exhibited 100% susceptibility to most of the tested antimicrobials including nalidixic acid and ciprofloxacin [23,28]. In addition, *Salmonella* isolates from reptile feces were reported to show a higher rate of susceptibility to quinolones [29]. In contrary, clinical strains isolated from humans and poultry could resist nalidixic acid (88%) and were less susceptible to ciprofloxacin (23%) [30]. According to the personal communication with pet shops, the studied group of turtles was juvenile and aged

less than 4 weeks. All the individuals were purchased soon after hatched, did not show any sign of disease and were not exposed to antibiotics. It could be the reason *S. enterica* subsp. *enterica* isolates to become more susceptible to quinolone antibiotics.

Previously, *qnrA*, *qnrB*, *qnrS*, and *aac(6)-Ib-cr* plasmid-mediated resistance genes were reported in quinolone-resistant, non-typhoidal *Salmonella* [36]. The Qnr protein induces resistance to nalidixic acid, thereby decreasing or limiting susceptibility to fluoroquinolones [31]. However, this study could not amplify any PMQR determinant while all the isolates were sensitive to tested quinolones. Studies reported *S. enterica* of poultry and human origin showing no *aac(6)-Ib-cr* in their plasmids despite they were resistant to quinolones [30,33]. Moreover, Asai *et al.* [34] could detect *qnrS* among 0.08% of the *S. enterica* isolates of food-producing animals while Kim *et al.* [33] reported *qnrB4* and *qnrS1* in 3.2% of the isolates from poultry. Prevalence of *qnr* genes in *S. enterica* human clinical isolates was also reported as low in recent studies [35,36].



**Figure 1.** Neighbor-joining phylogenetic tree derived by analyzing *S. enterica* subsp. *enterica gyrA* sequences obtained from the current study and downloaded from NCBI. Sequences referred to as AJ919282.1, GU330246.1, HQ448882.1, KP184391.1, FJ222660.1, HQ176368.1, KC121321.1, KP290113.1, and EU512996.1 were obtained from NCBI public database and the rest of the sequences were acquired from the current study. 1, 2-Major clads

In *Salmonella*, the most common point mutations associated with resistance to quinolones occur in the *gyrA* gene, resulting from substitutions of Ser-83 with Tyr, Phe, or Ala, and of Asp-87 with Asn, Gly, or Tyr [37]. In addition to above two sites, a recent study reported *gyrA* mutations of Ala-131 position in poultry and human isolates [30]. Another study could detect mutations in *parC* in addition to the *gyrA* mutations but in very low rate compared to *gyrA* [36]. Besides, Ser83-

Phe substitution was detected in *Salmonella* spp. isolated from poultry [33]. Although some non-specific and silent mutations of the amplified region were noticed, current study could not identify any of above mentioned specific point mutations within the amino acid positions 67 to 106. Therefore, it is reasonable to assume that QRDRs of every isolate in this study is less mutated compared to highly resistant isolates.

Phylogenetic analysis of *gyrA* sequences produced a

neighbor-joining tree showing 2 major clads referred to as 1 and 2. Interestingly, clad 1 comprised of isolates of the current study while clad 2 enclosing all clinical isolated both from human and animals. This outcome clearly interprets the genetic similarity of *S. enterica* subsp. *enterica* isolated from pet turtles and how they diverge from quinolone-resistant clinical isolates. Molecular phylogeny of *Salmonella* spp. has been investigated recently using *gyrA* region in which the antibiotic-resistant and sensitive isolates were separated into two different clusters [38].

In conclusion, it is plausible that the *S. enterica* subsp. *enterica* isolated from pet turtles are not resistant to tested quinolones and their QRDRs were genetically more conserved than that of quinolone-resistant strains. So it could be a good point for turtle keepers, but further studies concerned with more antimicrobials are highly recommended for a better understanding.

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**Conflict of interests** The authors declare that there is no financial conflict of interests to publish these results.

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