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Research Article
High Prevalence of Quinolone Resistance Genes in *Citrobacter freundii* Isolated from Pet Turtles

Sabrina Hossain, B.C.J. De Silva, S.H.M.P. Wimalasena, H.N.K.S. Pathirana and Gang-Joon Heo

Veterinary Medical Center, College of Veterinary Medicine, Chungbuk National University, Chungdae-ro 1, Seowon-gu, 28644 Cheongju, Chungbuk, Korea

Abstract

Background and Objective: *Citrobacter freundii* is a normal bacterial flora in pet turtles, which could opportunistically become pathogenic. Their possession of quinolone resistance genes owns significance both in humans and animals. Therefore, the aim of the study was to determine the quinolone resistance genes in *C. freundii* isolated from seven species of pet turtles. **Methodology:** Antimicrobial susceptibility was determined by disk diffusion test and minimum inhibitory concentration (MIC) values against quinolones. Transferrable quinolone resistance determinants such as plasmid-mediated quinolone resistance (PMQR) genes were identified by PCR. Nucleotide sequencing was performed to detect *aac(6')-Ib-cr* variant and point mutations in quinolone resistance determining region (QRDR) of *gyr*A gene. **Results:** Twenty-nine *C. freundii* isolates were obtained from 41 fecal samples of pet turtles. All the isolates were resistant against nalidixic acid in disk diffusion test. Each isolate from river cooter turtles showed a high quinolone resistance compared to others in disk diffusion test and MIC values. Four isolates from Chinese stripe-necked turtles showed reduced susceptibility to ciprofloxacin in MIC. With regards to PMQR determinants, the *qnr*B was the most prevalent gene (51.17%) among all isolates. The *qnr*S gene was present in seven *C. freundii* isolates (24.14%). The *aac(6')-Ib-cr* gene was detected only in four isolates (13.79%). A single amino acid substitution (Thr83-Ile) was observed in the *gyr*A gene of 8 (27.59%) isolates. **Conclusion:** The current study revealed that most of the *C. freundii* strains isolated from pet turtles are resistant to quinolones and harbored PMQR genes and QRDR mutations.

Key words: *Citrobacter freundii*, PMQR genes, QRDR mutations, pet turtles, public health

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Corresponding Author: Gang-Joon Heo, Veterinary Medical Center, College of Veterinary Medicine, Chungbuk National University, Chungdae-ro 1, Seowon-gu, 28644 Cheongju, Chungbuk, Korea Tel: +82-43-261-2617 Fax: +82-43-267-3150

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Citrobacter freundii is a Gram-negative opportunistic bacterium which is contemplated as one of the ordinary flora of the digestive tract of humans and other animals[1]. *Citrobacter freundii* is the etiological agent of several infections such as severe diarrhea, gastroenteritis, urinary tract infections, pneumonia, neonatal meningitis and brain abscesses like sporadic infections in humans[2,3].

Quinolone is the most popular antibacterial group used for both human and veterinary medical purposes. They are widely used in treating *C. freundii* infections[4,5]. Over-practicing of quinolones can be the main aspect leading to quinolone resistance[6]. Therefore, resistance to quinolones is a growing problem in *C. freundii* as well as in other bacteria of Enterobacteriaceae group[7].

In *C. freundii*, DNA gyrase is the main target of quinolones[8]. Mutations in DNA gyrase A or B subunits encoded by *gyr*A and *gyr*B cause quinolone resistance. Among them, *gyr*A alterations are well recognized. In *C. freundii*, mutations in quinolone resistance determining region (QRDR) are frequently located in the amino acid positions, Thr83 and Asp87, respectively[9]. Besides, like other Gram-negative bacteria, there are some secondary targets for quinolones in *C. freundii*, such as *par*C and *par*E subunits of topoisomerase IV[10].

Plasmid mediated quinolone resistance (PMQR) genes are substitutive agents of quinolone resistance that encode DNA gyrase protection proteins[11]. The first plasmid carrying quinolone gene, *qnr*A was documented in the year of 1998 in *Klebsiella pneumoniae*. After that, two *qnr* determinants, such as *qnr*B and *qnr*S were reported in other Enterobacteria[12]. The plasmids harboring the *qnr* genes are responsible for the horizontal transfer of quinolone resistance genes among the bacteria[13]. In addition to the *qnr* genes, another transferable gene has frequently been reported. The aminoglycoside acetyltransferase, *aac(6')-Ib-cr* gene shows the mechanism of reducing the susceptibility to quinolones by acetylation[14]. Like other Enterobacteriaceae, the *qnr* and *aac(6')-Ib-cr* genes have frequently been observed in clinical and environmental *C. freundii* strains[12].

Nowadays, turtles are being raised as pets globally. However, they are the hosts for different types of bacteria which are related to the infection in human and other warm-blooded animals[15]. Recently, human pathogenic *Salmonella* spp., was isolated from the healthy pet turtles in Korea[16]. *Citrobacter freundii* was also frequently reported both in healthy and diseased turtlep[17,18]. *Citrobacter freundii* is constantly in relation with reptiles, especially in infected turtles. It has been reported that *C. freundii* causes septicemic cutaneous ulcerative disease (SCUD). In fact, this disease can be dispersed among turtles in a captive condition within several days[19,20]. Furthermore, *C. freundii* isolated from pet turtles, turtle eggs and the pond water showed a high-level resistance to aminoglycosides, especially to gentamicin[21].

Nevertheless, genetics-based studies to characterize the quinolone resistance of pet turtle-borne *C. freundii* have not been conducted before. Therefore, the aims of this study were to examine the quinolone susceptibility, to screen the PMQR and QRDR resistant determinants in *C. freundii* isolated from seven popular varieties of pet turtles and raise awareness about the risk factors related to public health.

MATERIALS AND METHODS

Selection of turtle species: Forty one pet turtles of seven commercially popular species were bought from pet shops and online markets in Korea. The turtles were purchased with an average weight of 15±2 g, carapace diameter of 40±5 mm and were under 4 weeks of age. All of the turtles were healthy and no clinical signs of diseases were inspected. There was no antimicrobial therapy applied to the turtles. Among the 41 pet turtles, 2 alligator snapping turtles (*Macroclemys temminckii*), 2 African sideneck turtles (*Pelusios castaneus*), 14 Chinese stripe-necked turtles (*Ocadia sinensis*), 3 peninsula cooters (*Pseudemys peninsularis*), 10 river cooters (*Pseudemys concinna concinna*), 3 western painted turtles (*Chrysemys picta belli*) and 7 yellow-bellied sliders (*Trachemys scripta scripta*) were used for the study.

Isolation and identification of *C. freundii*: Fecal samples from the turtles were enriched in tetrathionate broth (MBcell Ltd., Seoul, Korea) by incubation at 37°C for 24 h. Enriched samples were streaked onto MacConkey agar (MBcell Ltd., Seoul, Korea) and incubated at 37°C for 24 h. Pinkish colonies assumed for *Citrobacter* spp., were subcultured onto xylose lysine deoxycholate agar (MBcell. Ltd, Seoul, Korea) and incubated at 37°C for 24 h. Several biochemical tests such as citrate test, indole test and H_2S test were performed to identify *C. freundii*. Species status of all bacterial isolates were confirmed by 16S rRNA gene sequencing at Cosmogenetech Co., Ltd. (Daejeon, Korea) using universal primers 518F and 800R.

Antimicrobial susceptibility testing: Susceptibility pattern of 29 *C. freundii* isolates were detected for nalidixic acid, ciprofloxacin and ofloxacin by disk diffusion test on

Mueller-Hinton agar (MBcell Ltd., Seoul, Korea). Minimum inhibitory concentrations (MIC) of nalidixic acid (1-512 mg L^{-1}), ciprofloxacin (0.125-64 mg L^{-1}) and ofloxacin (0.125-64 mg L^{-1}) were determined by broth microdilution method in a 96-well microtiter plate[22]. All susceptibility testing were conducted according to the recommendations of Performance Standards for Antimicrobial Susceptibility Testing; Clinical and Laboratory Standards Institute[23].

PCR amplification of *qnr* and *aac(6')-Ib* genes: The PCR was carried out to detect *qnr*A, *qnr*B, *qnr*S and *aac(6')-Ib* genes. The primer details were acquired from Cattoir *et al.*[24], Mammeri *et al.*[25] and Chenia[26] as shown in Table 1. The PCR mixture 25 µL, contained 12 µL Quick *Taq* HS DyeMix (Toyobo Co., Ltd., Japan), 9 µL PCR water, 1 µL DNA template and 1 µL of each primer. The thermal cycle for amplification of *qnr*B gene consisted of 10 min initial denaturation at 94°C, 32 repeated cycles of 45 sec at 94°C, 45 sec at 53°C, 1 min at 72°C and a final extension at 72°C for 10 min. The *qnr*A and *qnr*S genes were amplified using conditions; 95°C for 10 min, 35 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min and 72°C for 10 min. The PCR conditions for amplifying *aac(6')-Ib* were 94°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec and 72°C for 10 min with 34 repeated cycles. The PCR products were checked in 1.5% (W/V) agarose gel using gel loading buffer with DNA stain (Jena Bioscience GmbH, Germany). The amplicons were purified using Expin™ PCR SV purification kit (GeneAll, Korea) and sequenced by Cosmogenetech Co., Ltd. (Daejeon, Korea). Acquired *aac(6')-Ib* sequences were analyzed by comparing with the published sequences in GenBank database.

Detection of mutations in quinolone resistance determining region: The PCR was performed to detect mutations in QRDR of *gyr*A gene of *C. freundii* using previously described primers and conditions[25] (Table 1). The PCR products of *gyr*A gene were purified using Expin™ PCR SV kit (GeneAll, Korea) and sequenced by Cosmogenetech Co., Ltd. (Daejeon, Korea). Acquired sequences were subjected to detection of mutations by comparison with a previously reported *gyr*A reference sequence[27]. Analyzing and comparison of QRDR sequences were performed using Mutation Surveyor V5.0.1 (Softgenetics LLC, USA) software.

RESULTS

Isolation of *C. freundii* from pet turtles: Twenty nine *C. freundii* strains were isolated from 41 individual turtles as follows: (1) African sideneck turtle, (2) alligator snapping turtles, 11 Chinese stripe-necked turtles, 2 peninsula cooters, 8 river cooters, 2 Western painted turtles and 3 yellow-bellied sliders.

Quinolone resistance patterns in *C. freundii* isolates: The disk diffusion and MIC results are shown in Table 2. All tested isolates showed nalidixic acid resistance in disk diffusion test. The isolates from river cooters and Chinese stripe-necked turtles showed the distinctive quinolone resistance patterns. Five isolates from river cooters were resistant against all quinolones in disk diffusion test. Except for only three isolates from Chinese stripe-necked turtles which were intermediately resistant to ciprofloxacin, rest of the isolates obtained from the other turtle species showed susceptibility to ciprofloxacin and ofloxacin.

Meanwhile, 4 out of 8 isolates from river cooters exhibited higher MIC values for nalidixic acid, ciprofloxacin and ofloxacin. Bacterial strains isolated from the other turtle species with low MIC value were also noticed. Only four isolates from Chinese stripe-necked turtles showed higher MIC values against ciprofloxacin.

Detection of *qnr* and *aac(6')-Ib-cr* genes: The *qnr*B gene was found to be the most prevalent among *C. freundii* isolates. Thirteen from 29 (51.17%) strains were observed to

Table 1: Nucleotide sequence of oligonucleotide primers used in the study for detecting quinolone resistance genes

Primers	Nucleotide Sequence	Size of the fragment (bp)	Targeted gene
QnrAm-F	AGAGGATTTCTCACGCCAGG	580	*qnr*A
QnrAm-R	TGCCAGGCACAGATCTTGAC		
QnrB-F	GATCGTGAAAGCCAGAAAGG	496	*qnr*B
QnrB-R	ACGATGCCTGGTAGTTGTCC		
QnrSm-F	GCAAGTTCATTGAACAGGGT	428	*qnr*S
QnrSm-R	TCTAAACCGTCGAGTTCGGCG		
GyrA6-F	CGACCTTGCGAGAGAAAT	626	*gyr*A
gyrA316-R	GTTCCATCAGCCCTTCAA		
aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	482	*aac(6')-Ib*
aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT		

Table 2: Quinolone resistance patterns of *C. freundii* isolated from pet turtles and their quinolone resistance gene contents

	Disk diffusion zone diameters (mm)[b]			MIC (μg/mL)[b]					
Isolate[a]	NA30	CIP5	OFX5	NA	CIP	OFX	*qnr* gene content	*aac(6')-Ib-cr*	*gyrA* mutations
AF1	0(R)	34(S)	29(S)	2(S)	≤0.125(S)	0.125(S)	*qnrB*	-	-
CSN1	0(R)	38(S)	36(S)	4(S)	≤0.125(S)	0.125(S)	-	-	-
CSN2	0(R)	42(S)	32(S)	4(S)	≤0.125(S)	0.250(S)	-	-	-
CSN3	0(R)	40(S)	26(S)	8(S)	≤0.125(S)	≤0.125(S)	*qnrB*	-	-
CSN4	0(R)	37(S)	40(S)	4(S)	≤0.125(S)	≤0.125(S)	-	-	-
CSN5	0(R)	35(S)	32(S)	8(S)	2(I)	0.250(S)	*qnrB*	-	-
CSN6	0(R)	20(I)	30(S)	4(S)	2(I)	0.250(S)	*qnrS*	*aac(6')-Ib-cr*	-
CSN7	0(R)	36(S)	40(S)	4(S)	≤0.125(S)	≤0.125(S)	-	-	-
CSN8	0(R)	20(I)	20(S)	≤1(S)	2(I)	0.125(S)	-	-	-
CSN9	0(R)	20(I)	30(S)	2(S)	4(R)	0.125(S)	-	-	-
CSN10	0(R)	40(S)	32(S)	4(S)	≤0.125(S)	≤0.125(S)	*qnrB*	-	-
CSN11	0(R)	40(S)	30(S)	≤1(S)	≤0.125(S)	≤0.125(S)	-	-	-
PC1	0(R)	35(S)	32(S)	2(S)	0.500(S)	≤0.125(S)	-	-	-
PC2	0(R)	34(S)	34(S)	4(S)	≤0.125(S)	≤0.125(S)	-	-	-
RC1	0(R)	31(S)	29(S)	64(R)	4(R)	4(I)	*qnrS*	-	-
RC2	0(R)	15(R)	0(R)	64(R)	4(R)	2(S)	-	-	-
RC3	0(R)	31(S)	14(I)	64(R)	4(R)	1(S)	-	-	Thr83-Ile
RC4	0(R)	0(R)	0(R)	64(R)	8(R)	8(R)	*qnrS*	-	Thr83-Ile
RC5	0(R)	0(R)	0(R)	64(R)	16(R)	8(R)	*qnrB, qnrS*	*aac(6')-Ib-cr*	-
RC6	0(R)	20(I)	0(R)	64(R)	4(R)	2(S)	*qnrS*	-	Thr83-Ile
RC7	0(R)	0(R)	0(R)	64(R)	64(R)	32(R)	*qnrB, qnrS*	*aac(6')-Ib-cr*	Thr83-Ile
RC8	0(R)	0(R)	8(R)	64(R)	8(R)	8(R)	*qnrB*	-	Thr83-Ile
SN1	0(R)	37(S)	29(S)	4(S)	≤0.125(S)	≤0.125(S)	*qnrB, qnrS*	-	Thr83-Ile
SN2	0(R)	31(S)	29(S)	8(S)	≤0.125(S)	≤0.125(S)	*qnrB*	-	-
WP1	0(R)	39(S)	32(S)	4(S)	≤0.125(S)	0.125(S)	*qnrB*	-	-
WP2	0(R)	37(S)	31(S)	4(S)	≤0.125(S)	0.250(S)	*qnrB*	-	-
YB1	0(R)	34(S)	33(S)	4(S)	0.250(S)	0.250(S)	*qnrB*	-	-
YB2	0(R)	36(S)	27(S)	8(S)	0.500(S)	0.500(S)	*qnrB*	*aac(6')-Ib-cr*	Thr83-Ile
YB3	0(R)	38(S)	36(S)	4(S)	≤0.125(S)	0.125(S)	-	-	Thr83-Ile

[a]*C. freundii* isolates from African sideneck turtle, Chinese stripe-necked turtle, peninsula cooter, river cooter, Western painted turtle, alligator snapping turtle and yellow-bellied slider were indicated as AF, CSN, PC, RC, SN, WP and YB, respectively. [b]Disk diffusion zone diameters (mm): NA30: Nalidixic Acid (30 μg), CIP5: Ciprofloxacin (5 μg) and OFX5: Ofloxacin (5 μg), S: Susceptible, I: intermediate, R: Resistant were designated using breakpoints described by the Clinical Laboratory Standards Institute (CLSI)[23]. The MIC (μg mL^{-1}): S: Susceptible, I Intermediate, R Resistant were designated using breakpoints described by the Clinical Laboratory Standards Institute (CLSI)[23] following MIC determination with broth microdilution method[22]

harbor *qnr*B gene. On the contrary, the *qnr*S gene was found in seven isolates (24.14%). Only four isolates (13.79%) contained *aac(6')-Ib-cr* gene. Yet, *qnr*A gene was not detected in any of the isolates (Table 2). The *qnr* and *aac(6')-Ib-cr* genes were frequently encountered in the strains from river cooters.

Mutation determination in QRDR region of *gyr*A gene: Mutations in QRDR region of *gyr*A gene was detected in 8 out of 29 (27.59%) isolates (Table 2). Among them, five strains that showed less susceptibility to quinolones in disk diffusion and MIC test were occupied with *gyr*A alterations resulting in the amino acid substitution of Thr83-Ile. Three isolates having low MIC values to quinolones also had a single mutation in the same amino acid position (Thr83-Ile) of *gyr*A gene.

DISCUSSION

As an opportunistic pathogen, *C. freundii* has been found to cause several infections both in humans and animals. In the meantime, it has been known to develop antimicrobial resistance, especially to quinolones. Thus, the study about antimicrobial resistance focusing the resistance of *C. freundii* against quinolones owns significance.

The current study could characterize *C. freundii* isolates from pet turtles showing both resistance and susceptibility to quinolones. With regards to *qnr* gene characterization, the isolates carrying *qnr*B were found in both resistant and susceptible *C. freundii* isolates. Similar results were observed in the previous studies[6,12,28]. The *qnr*B was found most frequently in *C. freundii* than other Enterobacteriaceae. About 75% of *qnr*B alleles were discovered in the *C. freundii* isolates and most of them were isolated from human clinical cases[29]. Furthermore, the *qnr*S gene was mostly present in the isolates having higher MIC values against quinolones while some other studies also noted that the *qnr*S positive human isolates were susceptible to nalidixic acid and ciprofloxacin[28,30].

Interestingly, the *aac(6')-Ib-cr* gene was found in the isolates that were either less susceptible to quinolones in MIC and disk diffusion test or harboring at least one or two qnr

genes. Besides, the strains carrying both *qnr*S and *aac(6')Ib-cr* were highly resistant against tested quinolones. On the other hand, the majority of the *qnr*S and *aac(6')-Ib-cr* positive strains were isolated from river cooters and Chinese stripe-necked turtles. As the *qnr*S and *aac(6')Ib-cr* genes are predominant in the plasmid of these bacterial strains, the possibility of horizontal transfer of these genes between the bacterial flora of turtles and human should not be under valued. Although the presence of *qnr*A in *C. freundii* was recorded by Yang *et al.*[31], none of the isolates showed *qnr*A gene in this study. Limitations in the transmission of *qnr*A between organisms could be the reason as a previous study indicated[30]. Also, another study revealed that the increasing rate of *qnr*A gene may not cause the high resistance level of quinolones[32].

The mutations in QRDR of *gyr*A gene were note worthy in current study. The alteration in *gyr*A is a generic feature of quinolone resistance in bacteria and most of the alterations are located in the first half of *gyr*A[10]. Particularly, the amino acid codons 83 and 87 in *gyr*A gene displayed typical alterations in clinical isolates[13]. According to Navia *et al.*[33], *C. freundii* strains which were having Thr83-Ile alteration exhibited a higher level of resistance to quinolones. It has been identified that the Thr83-Ile mutation arises due to the substitutions at the nucleotide 248th position in *gyr*A. The study noted that a single alteration in position 83 of *gyr*A is adequate for establishing a high level of nalidixic acid resistance (MIC$\geq$024 μg). Another study indicated that single amino acid alteration in *gyr*A caused a greater level of ciprofloxacin resistance[8]. The present study could find five *C. freundii* isolates which were highly resistant to ciprofloxacin (MIC 4-64 μg mL^{-1}) and nalidixic acid (MIC 64 μg mL^{-1}) showing Thr83-Ile *gyr*A mutation.

Nevertheless, a few isolates were susceptible to ciprofloxacin and nalidixic acid in MIC test although they have the mutation in *gyr*A. The same outcome was encountered in detecting *qnr* genes in which the possession of *qnr* genes was not limited to resistant strains. Even though it is controversial, similar results have been reported in *C. freundii* and also in *Aeromonas* spp., pointing out the necessity of investigating the organismal internal factors and mechanisms which could suppress or alter the gene expression[25,26].

CONCLUSION

It can be concluded that pet turtle-borne *C. freundii* strains are a reservoir of chromosomal and plasmid-mediated quinolone resistance determinants. The prevalence of transferable quinolone resistance genes in these bacterial strains play a significant role both in human and anima medicine. Therefore, further studies focusing on characterization of resistance genes related to other antimicrobial groups in *C. freundii* from pet turtles are highly recommended.

SIGNIFICANCE STATEMENT

The prevalence of quinolone resistance genes in the *C. freundii* isolates from pet turtles are alarming their prospective pathogenicity leading into public health concerns.

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