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Research Article
Virulence Factors and Antimicrobial Resistance Pattern of *Citrobacter freundii* Isolated from Healthy Pet Turtles and their Environment

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Abstract

Background: Zoonotic *Citrobacter freundii* infection can occur if pet turtle owners fail to hygienically handle the turtle or the turtle's environment. Therefore, the virulence characteristics and resistance patterns of *C. freundii* to commonly used antimicrobials should be understood. **Methodology:** *Citrobacter freundii* isolates were characterized by conventional pathogenicity tests, such as proteolysis, biofilm formation and hemolysis, PCR assays of virulence genes and antimicrobial disk diffusion tests. **Results:** Forty seven presumptive *C. freundii* isolates obtained from 41 fecal and 18 environmental samples including water and soil samples were confirmed as *C. freundii* by biochemical tests and 16S rRNA gene sequencing. Proteolysis and biofilm formation were shown in 17 and 6 isolates, respectively. No isolates showed hemolysis. The PCR assay for the presence of *slt-II* or *slt-II* related genes and *via B* genes were successful in 2 and 4 isolates, respectively. In the antimicrobial susceptibility test, most isolates were susceptible to all tested antibiotics except ampicillin, amoxicillin, cephalothin, cefoxitin and nalidixic acid. Non-susceptible isolates to penicillins (piperacillin and ticarcillin), fluoroquinolones (ciprofloxacin and norfloxacin), aminoglycosides (gentamicin) and other antibiotics (trimethoprim/sulfamethoxazole) were frequently observed among the isolates. A few isolates were resistant to imipenem, aztreonam, ceftriaxone and cefotaxime. **Conclusion:** In conclusion, it can be said that pet turtles are a potential public health risk due to the virulence and antimicrobial resistance of *C. freundii*.

Key words: *Citrobacter freundii*, pet turtles, virulence factors, antimicrobial resistance, public health

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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, aerobic or facultative and rod shaped bacterium which is considered a commensal species of the intestinal tract of humans and other animals[1]. *Citrobacter freundii* is associated with several opportunistic infections, such as severe diarrhea, urinary tract infections, pneumonia, neonatal meningitis and brain abscessesin humans. Neonatal meningitis can be fatal with a 25-50% mortality rate among infants. However, *Citrobacter freundii* related infections are generally limited to infants and immuno-compromised people[2,3]. Despite this, certain virulence factors can be acquired by *Citrobacter freundii* strains that enable them to cause infections in humans. The major virulence factors found in diarrhea-associated *Citrobacter freundii* are toxins. According to Schmidt *et al.*[4], *Citrobacter freundii* can acquire *slt-II* or *slt-II* related shiga-like toxins or capsular polysaccharide virulence antigen, such as *via B* gene, which is the most common virulence factor found in *Salmonella enteric* serovar typhi and *S. enteric* serovar paratyphi and causes typhoid-like diseases[5]. Other *Citrobacter* related virulence factors including proteolysis, hemolysis and biofilm formation have also been previously observed[6]. For example, diarrheagenic biofilm formation was discovered in *Citrobacter freundii*[7].

Citrobacter freundii is ubiquitous in humans and animals. *Citrobacter freundii* is also prevalent in soil and water through contamination from the waste materials of animals. *Citrobacter freundii* is also persistently linked with reptiles, particularly in turtle infections. It is known as the etiological agent of Septicemic Cutaneous Ulcerative Disease (SCUD), which is characterized by anorexia, lethargy, liver necrosis and petechial hemorrhages on the external part of the body. This disease can be horizontally transmitted to other turtles in a captive condition within several days[8,9].

Currently, reptiles rearing has become more popular throughout the world. Pet turtles are the most popular among them because of their cute appearance and complaisant nature. However, turtles can carry numerous types of bacteria that have been associated with zoonotic infection of warm blooded animals[10]. Previous reports have focused on salmonellosis caused by contact with pet turtles[11]. In a similar fashion, *Citrobacter freundii* can cause human infections if pet owners do not maintain proper hygiene when touching their turtles. Bacteria can thereby be transferred through direct physical contact with the infected turtles or through contaminated environments, such as the soil and watering turtle cages. Some countries have therefore passed regulations concerning this issue. Since, 1975, the U.S. Food and Drug Administration[12] banned the distribution of small turtles with a carapace smaller than 4 inches to prevent horizontal transmission of pathogenic bacteria from turtles to small children. However, there are no such regulations in Korea although the pet turtle industry is widespread.

Prevention of *Citrobacter freundii* infection can be achieved through hygienic handling of turtles and proper cage management. However, failure to meet those conditions may result in *Citrobacter freundii* infections. In that case, antimicrobial treatment must be applied, but previously successful antimicrobials may be overused and cause *Citrobacter freundii* to evolve resistance, therefore, treatment may prove unsuccessful because of antimicrobial resistance of *Citrobacter freundii*[13].

This study is aimed to identify and characterize the *Citrobacter freundii* in pet turtles according to their virulence and antibiotic resistance patterns to determine the risk to public health.

MATERIALS AND METHODS

Purchase of pet turtles: Forty one aquatic pet turtles of 7 commercially popular species were randomly purchased 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 in healthy condition and noclinical signs of diseases were observed.

Among the 41 turtles, 2 alligator snapping turtles (*Macroclemys temminckii*), 2 African side-neck 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 (*Chrysemyspicta belli*) and 7 yellow belly sliders (*Trachemys scripta scripta*) were studied.

Raising condition of pet turtles: Nine cages each containing 3-7 turtles of the same species from the same pet shop or online market were managed, each cage contained a slope made from soil and pebbles, 2 L of sterilized water and a canister filter to maintain water quality. The turtles were raised following the general husbandry method[14]. Gammarus dried shrimp with calcium supplements (Samhotech Co., Ltd., Seoul, Korea) were fed twice a day, while water temperature was kept within 26±2°C, pH 6.5-8.2 and 12 h of photoperiod each day were maintained during the experiment.

Sample collection: Each turtle was placed in a 500 mL beaker with 5 mL of sterile distilled water for 24 h. About 1 mL of the

distilled water containing the feces of turtles was taken as a fecal sample. Fecal samples were taken on the 1st day after purchasing. Poly-gloves were changed to avoid bacterial contamination. The samples from the turtle's environment were taken after 7 days. Five grams of soil from a turtle's rest or feeding area were obtained using a sterile spoon. About 10 mL of water was collected from each cage with a sterile pipette.

Enrichment and isolation of *Citrobacter freundii*: About 1 mL of water or 1 g of soil was submerged in 9 mL of tetrathionate broth (MBcell Ltd., Seoul, Korea) at 37°C for 24 h. After incubation, the broth was mixed for 5 sec with a vortex agitator. Then one loop full from each tube was streaked onto a plate of MacConkey agar (MBcell Ltd., Seoul, Korea) and incubated at 37°C for 24 h. Pinkish colonies with a glossy surface were suspicious for *Citrobacter* spp., were therefore smeared onto a plate of xylose lysine deoxycholate agar (MBcell. Ltd, Seoul, Korea) and incubated at 37°C for 24 h.

Biochemical tests and 16S ribosomal RNA gene sequencing: To characterize bacterial isolates from the feces, water and soil, several biochemical tests were carried out. A citrate test was performed by streaking a bacterial sample on simmons citrate agar (MBcell Ltd., Seoul, Korea) and incubating at 37°C for 24 h. A motility, H_2S and indole test were also performed by streaking isolates on sulfide indole motility medium (MBcellLtd, Seoul, Korea) and incubating at 37°C for 24 h[15,16]. All bacterial isolates from the fecal samples were 16S rRNA sequenced using universal sequencing primers 518 F and 800 rat Cosmogenetech Co., Ltd. (Seoul, Korea). The resulting sequences were analyzed and compared with 16S rRNA gene sequences stored in GenBank.

Detection of virulence factors

Conventional pathogenicity tests: All of the isolated *Citrobacter freundii* were inoculated onto 5% sheep blood agar (MBcell Ltd., Seoul, Korea) and incubated at 37°C overnight to examine the hemolysis pattern. Proteolysis was examined by observing the presence of complete lysis around the colony and a clearing on the medium[6]. According to the procedures of Niveditha *et al.*[17] and Christensen *et al.*[18], all bacterial isolates were incubated in conical plastic test tubes (SPL Life Sciences, Korea) containing tryptic soy broth (MBcell Ltd., Seoul, Korea) at 37°C for 3 days of incubation to investigate biofilm formation.

Detection of virulence genes: The Polymerase Chain Reaction (PCR) was used to detect *via B* and *slt-I* or *slt-II* associated virulence genes in the isolated bacterial strains. The primers used were obtained from Schmidt *et al.*[4] and Lin *et al.*[5] are shown in Table 1. The PCR was conducted by Cosmogenetech, Co., Ltd, Korea.

The PCR mixture contained 0.5 µL of Taq polymerase, 10×SP Taq buffer, 2 µL of dNTP, 5.0 µL tuning buffer, 1.0 µL template and 12 µL dissolved water and 1 µL of each primer pairs. The DNA for *slt-I* or *slt-II* related toxin genes were amplified by GK1/GK4 primer pairs in a thermal cycler using the following process: 95°C for 5 min and 30 cycles of 95°C for 30 sec, 54°C for 30 sec and 72°C for 1 min 30 sec and 72°C for 10 min[4]. The thermal cycle for amplification of *via B* gene consisted of 5 min initial denaturation at 95°C, later 95°C for 30 sec, 62°C for anneal temperature up to 30 sec, 1 min elongation period at 72°C and at last final extension was done at 72°C for 10 min[5]. The PCR products in the agarose gel were stained with ethidium bromide (0.5 mg mL^{-1}) and observed. The final PCR bands were photographed after observation under ultraviolet light.

Antimicrobial resistance test: All 47 isolates were subjected to disk diffusion testing with 19 antimicrobial agents used against Gram-negative bacteria suggested by World Organisation for Animal Health[19]. The disk diffusion test was carried out according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards[20]. All *Citrobacter freundii* were cultured on tryptic soy agar (MBcell Ltd., Seoul, Korea) and generated colonies were adjusted to a turbidity of McFarland 0.5 (5×10^5 CFU mL^{-1}) standard by adding saline. Then the suspension was spreadonto Mueller-Hintonagar (MBcell Ltd., Seoul, Korea). Amoxicillin (30 µg), ampicillin (10 µg) and nalidixic acid (30 µg) disks were prepared by soaking each paper disk with sterile pipet tube with antimicrobial. Disks containing amikacin (30 µg), aztreonam (30 µg), cefoxitin (30 µg), cefotaxime (30 µg),

Table 1: Sequence of oligonucleotide primers used in this study to detect virulence genes

Primers	Nucleotide sequence	Size of fragment (bp)	Expected nature of amplified product
VIAB-1	TGTCGAGCAGATGGATGAGCAT	516	*via B* gene
VIAB-2	ACGGCTGAAGGTTACGGACCGA		
GK1	COGGATCCATGAAGTGTATATTATTTAAATGG	1,260	Complete operon of
GK4	CCCGAATTCTCAGTCATTATTAAACTGCAC		*slt-I or slt-II* related toxin genes

ceftriaxone (30 μg), cephalothin (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), norfloxacin (10 μg), streptomycin (10 μg), tobramycin (10 μg), piperacillin (100 μg), ticarcillin (75 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg) were purchased from Oxoid Co., Ltd. (Seoul, Korea) and Kisan Biotech Co., Ltd. (Seoul, Korea). Five to seven disks were placed per each Mueller-Hinton agar plate and the plates were incubated for 24 h at 27°C. After incubation, antimicrobial resistance was detected by measuring the diameter of the inhibition zones formed on the plates.

RESULTS

Isolation and identification of *Citrobacter freundii*: The genus *Citrobacter* was differentiated according to colony morphology including yellow appearance on xylose lysine deoxycholate agar and pink colonies on MacConkey agar. Around 47 suspicious bacteria were presumptively identified as *Citrobacter freundii* from fecal, soil and water samples of turtles on the basis of several biochemical tests. All of the bacteria were then genetically confirmed as *Citrobacter freundii* by 16S rRNA gene sequencing. Table 2 shows the distribution of isolated *Citrobacter freundii* with respect to turtle species and source. Twenty nine *Citrobacter freundii* strains were isolated from fecal samples of the following turtles: 1 African side-neck turtle, 2 alligator snapping turtles, 11 Chinese stripe-necked turtles, 2 peninsula cooters, 8 river cootesr, 2 Western painted turtles and 3 yellow belly turtles. The other 18 *Citrobacter freundii* strains were isolated from soil and water samples from the turtle cages.

Detection of virulence factors: Table 3 shows the results of the conventional and molecular methods of virulence factor identification. Seventeen of 47 isolates showed proteolytic activity on skim milk agar. None of the isolates showed a typical hemolysis pattern on 5% sheep blood agar plates. However, 6 isolates tested positive for biofilm formation in the tube adherence test. In the PCR assays for virulence genes, only 2 isolates showed positive bands on PCR for *slt-I* and *slt-II* related virulence genes. In addition, 4 isolates were positive for the *via B* virulence gene.

Antimicrobial resistance test: The disk diffusion results are shown in Table 4. Clinical Standards Institute Standards[21] were followed as the interpretive criteria for selected antimicrobials. At least one isolate showed resistance to every antimicrobial except amikacin and meropenem. Every isolate showed antimicrobial resistance against amoxicillin, ampicillin,

Table 2: Distribution of *C. freundii* (n = 47) isolated from pet turtles and their environment

Name of turtles (No. of turtles)	Source (No. of isolates)
African side-neck turtle (n = 2)	Feces (1), soil (1) and water (1)
Alligator snapping turtle (n = 2)	Feces (2), soil (1) and water (1)
Chinese stripe-necked turtle (n = 14)	Feces (11), soil (2) and water (2)
Peninsula cooter (n = 3)	Feces (2), soil (1) and water (1)
River cooter (n = 10)	Feces (8), soil (2) and water (2)
Western painted turtle (n = 3)	Feces (2), soil (1) and water (1)
Yellow belly slider (n = 7)	Feces (3), soil (1) and water (1)

Table 3: Virulence factors of *Citrobacter freundii* isolated from all the fecal and environmental samples of pet turtles

Isolate	Proteolysis	Hemolysis	Bio-film formation	Virulence genes
AF1	-	-	-	*via B*
ES1	-	-	-	-
EW1	-	-	-	-
SN1	-	-	+	-
SN2	-	-	-	-
ES2	-	-	-	-
EW2	-	-	-	-
CSN1	-	-	-	-
CSN2	-	-	-	-
CSN3	-	-	-	-
CSN4	+	-	-	-
CSN5	-	-	-	-
CSN6	-	-	-	-
CSN7	+	-	-	-
CSN8	+	-	-	*via B*
CSN9	+	-	-	-
CSN10	-	-	-	-
CSN11	+	-	-	-
ES3	-	-	-	-
ES4	+	-	-	-
EW3	-	-	-	-
EW4	+	-	-	-
PC1	+	-	-	*via B*
PC2	-	-	-	*via B*
ES5	-	-	-	-
EW5	+	-	-	-
RC1	-	-	+	-
RC2	-	-	-	-
RC3	+	-	+	*slt-I*/or *slt-II* related
RC4	+	-	-	*slt-I*/or *slt-II* related
RC5	-	-	-	-
RC6	-	-	+	-
RC7	+	-	-	-
RC8	-	-	-	-
ES6	+	-	-	-
ES7	+	-	-	-
EW6	-	-	+	-
EW7	-	-	-	-
WP1	+	-	-	-
WP2	-	-	-	-
ES8	-	-	-	-
EW8	-	-	-	-
YB1	+	-	-	-
YB2	+	-	+	-
YB3	-	-	-	-
ES9	-	-	-	-
EW9	-	-	-	-

Table 4: Distribution of Susceptible (S), Intermediate (I), Resistance (R) strains of *Citrobacter freundii* isolates from pet turtles and their environment

	No. of *Citrobacter freundii* isolates		
Antimicrobials	R	I	S
Penicillins			
Ampicillin (10 µg)	47	0	0
Amoxicillin (30 µg)	47	0	0
Piperacillin (100 µg)	9	2	36
Ticarcillin (75 µg)	12	2	33
Cephalosporins			
Cephalothin (30 µg)	47	0	0
Cefotaxime (30 µg)	1	0	46
Ceftriaxone (30 µg)	1	0	46
Cefoxitin (30 µg)	46	1	0
Aminoglycosides			
Amikacin (30 µg)	0	0	47
Gentamicin (10 µg)	3	1	43
Streptomycin (10 µg)	9	7	31
Tobramycin (10 µg)	5	2	40
Carbapenems			
Imipenem (10 µg)	3	2	42
Meropenem (10 µg)	0	0	47
Fluroquinolones			
Ciprofloxacin (5 µg)	5	4	38
Norfloxacin (10 µg)	7	0	40
Others			
Aztreonam (30 µg)	1	0	46
Nalidixic acid (30 µg)	47	0	0
Trimethoprim/sulfamethoxazole (1.25/13.75 µg)	11	0	36

Inhibition zone diameters were evaluated according to CLSI[21] standards

cephalothin and nalidixic acid as expected. Nine isolates showed resistance to piperacillin and 12 against ticarcillin, respect. All were resistant against cefoxitin except one isolate. However, resistance against aminoglycosides and fluoroquinolone groups were observed in only small numbers of isolates. About 11 *Citrobacter freundii* strains were resistant against trimethoprim/sulfamethoxazole. One aztreonam resistant and three imipenem resistant isolates were identified.

DISCUSSION

According to the American Veterinary Medical Association[22], the increasing of popularity of turtle rearing has also increased the occurrence of zoonotic bacteria related infections. The percentage of US households with domestic turtles increased from 0.5-11% from 1996-2011. The U.S. Food and Drug Administration[12] has warned citizens that pet turtles are a potential source of pathogenic bacteria that are particularly risky for immuno-suppressed persons. Furthermore, they explained that turtle owners experienced bacterial infections because of improper hygiene and careless cage management.

In this study, *Citrobacter freundii* was isolated from 47 of 59 fecal and environmental samples of pet turtles. The isolation number was relatively high though they were all isolated from healthy turtles. However, healthy turtles are a silent carrier of pathogenic bacteria because they may shed bacteria with their feces. In a recent study, *Citrobacter freundii* was isolated from healthy pond sliders (*Trachemys scripta*) with a low isolation rate[23]. In this case, the fecal samples were taken immediately after purchasing the turtles to avoid horizontal contamination of bacteria through feces. After 7 days of rearing, the turtle's environments were contaminated through bacteria shedding. In addition, even in the small volume of aquaria water the turtle's feces contaminated the environment despite strict management of the aquaria.

We found several virulence factors in the isolated *Citrobacter freundii*. Seventeen isolates were positive for protease production, which allows them to hydrolyze the peptide bonds of essential proteins in eukaryotes, particularly in humans although the protease enzymes only infrequently serve as toxic factors to the host[24]. The lack of hemolytic inability indicates that our isolates could not break down red blood cells and cause hemolytic uremic syndrome in human[25]. On the other hand, six isolates were found to be able to form biofilm. In general, biofilm production is related to persistent infections which respond weakly to regular-antibiotic therapy. This helps to extent the antimicrobial resistant traits in nosocomial pathogens by expanding mutation rates and exchange of antimicrobial resistance genes[17]. Previous studies detected biofilm formation in clinical samples from patients[26]. Our discovery of biofilm producing bacteria from healthy pet turtles thereby demonstrates a potential public health risk.

The prevalence of *via B* and *slt-I/* or *slt-II* related genes in the bacterial isolates were very low in this study. The presence of *via B* gene allows the bacteria to evade the natural immune system by extending the host response during the infection[27]. There is also clear evidence of the presence of shiga like toxins in some strains of *Citrobacter freundii*. This contrasts with a previous study indicating that *Citrobacter freundii* isolated from animal sources contained no shiga like toxin genes[28]. That study also demonstrated a strain which had highly cytotoxicity to Hep-2 cells despite expressing no *slt-I/* or *slt-II* related toxin genes. Overall, the isolated strains of *Citrobacter freundii* that expressed virulent factors detected through conventional and PCR methods have the potential to act as zoonotic pathogens.

Antimicrobial multiresistance is another common problem concerning nosocomial bacteria like *Citrobacter freundii*. In this study, antimicrobial resistance testing of the isolates revealed that most of the strains were resistant to themajority of clinically used antimicrobial agents[13]. Most of the isolates showed resistance against penicillins and cephalosporins, which occurs due to the production of β-lactamase by *Citrobacter freundii*. Therefore, aminoglycosides, fluoroquinolones, fourth-generation cephems and carbapenems are considered the best antimicrobial choices for treating *Citrobacter freundii* infections[29]. However, this study isolated strains with resistance against streptomycin and tobramycin.

In addition, some *Citrobacter freundii* showed resistance against trimethoprim/sulfamethoxazole. This resulted from the presence of sulfamide and trimethoprim resistant traits as observed in a previous study[30]. In another study, ciprofloxacin, a fluoroquinolone was quite active against *Citrobacter freundii* isolated from fish. However, we isolated a few *Citrobacter freundii* strains with resistance tofluoroquinolones[31]. Though all strains were susceptible to amikacin and meropenem, resistance of some isolate stoaztreonam and imipenem revealed the capacity of *Citrobacter freundii* to adapt antimicrobial resistance. In the present study, amikacin was shown to act as the most effective drug tested, which is supported by previous studies[32,33]. Public awareness of the risk of zoonotic infection of *Citrobacter freundii* from pet turtles is essential to prevent expansion of multiple antimicrobial resistance.

CONCLUSION AND FUTURE RECOMMENDATIONS

This study concluded that healthy pet turtles are a potential carrier of *Citrobacter freundii* and strains could infect humans through direct contact with the turtle or careless management of the turtle's environment. The virulence factors and antimicrobial resistance patterns of this bacteria expose significant public health risk factors. Further study should be focused on genetic characterization of *Citrobacter freundii* in turtles and identification of antimicrobial resistant genes by molecular methods.

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