



Potential enterotoxicity and antimicrobial resistance pattern of *Aeromonas* species isolated from pet turtles and their environment

S. H. M. P. WIMALASENA^{1) #}, Gee-Wook SHIN^{2) #}, Sabrina HOSSAIN¹⁾ and Gang-Joon HEO^{1) *}

¹⁾Veterinary Medical Center and College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Korea

²⁾Bio-Safety Research Institute and College of Veterinary Medicine, Chonbuk National University, Jeonju 54596, Korea

ABSTRACT. To investigate the potential enterotoxicity and antimicrobial resistance of aeromonads from pet turtles as a risk for human infection, one hundred and two *Aeromonas* spp. were isolated from the feces, skin and rearing environments of pet turtles and identified by biochemical and *gyrB* sequence analyses. *Aeromonas enteropelogenes* was the predominant species among the isolates (52.9%) followed by *A. hydrophila* (32.4%), *A. dharkensis* (5.9%), *A. veronii* (4.9%) and *A. caviae* (3.9%). Their potential enterotoxicities were evaluated by PCR assays for detecting genes encoding cytotoxic enterotoxin (*act*) and two cytotoxic enterotoxins (*alt* and *ast*). 75.8% of *A. hydrophila* isolates exhibited the *act*⁺/*alt*⁺/*ast*⁺ genotype, whereas 94.4% of *A. enteropelogenes* isolates were determined to be *act*⁻/*alt*⁻/*ast*⁻. In an antimicrobial susceptibility test, most isolates were susceptible to all tested antibiotics except amoxicillin, ampicillin, cephalothin, chloramphenicol and tetracycline. Non-susceptible isolates to penicillins (ampicillin and amoxicillin) and fluoroquinolones (ciprofloxacin and norfloxacin) were frequently observed among the *A. enteropelogenes* isolates. Few isolates were resistant to imipenem, amikacin, ceftriaxone and cefotaxime. Collectively, these results suggest that pet turtles may pose a public health risk of infection by enterotoxigenic and antimicrobial resistant *Aeromonas* strains.

KEY WORDS: *Aeromonas* species, antimicrobial resistance, enterotoxin genes, *gyrB*, pet turtles

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For the past several decades, industries associated with pet animals have flourished due to social trends including increases in single-person households, elderly households and a low birthrate. While dogs and cats are considered traditional pets, exotic pets including tropical fish and reptiles have gained popularity. In particular, reptiles have become prominent in developed countries. However, reptile rearing poses a public health risk due to exposure to pathogens, such as *Salmonella* spp. and *Campylobacter* spp. through direct contact with either a healthy reptile or its environment [9, 18]. For example, turtles carry *Salmonella* species in their normal intestinal flora. However, most studies have focused on turtle-associated salmonellosis and characterization of that pathogen. Beyond *Salmonella* spp., there is little information about other pathogens contracted from pet reptiles and their environment.

The genus *Aeromonas* is composed of Gram-negative, facultative anaerobic, rod-shaped bacteria and consists of two groups: non-motile psychrophilic *Aeromonas salmonicida* and mesophilic motile *Aeromonas* species. *A. salmonicida* is a primary pathogen for fish, whereas mesophilic motile *Aeromonas* spp. have been associated with diseases in both warm and cold-blooded animals [10]. In addition, the latter are ubiquitous and autochthonous aquatic bacteria distributed worldwide, and are part of the normal microbial flora of many aquatic animals, such as fish, amphibians and reptiles. They can cause ulcerative stomatitis, pneumonia, dermatitis and septicemia in reptiles under stressful conditions, such as trapping, handling and thermoregulation [14]. Several countries have received serious setbacks in their turtle markets, because of *Aeromonas* spp. In Italy, severe outbreaks of *A. hydrophila* infection have been recorded among captive turtles, which led to massive mortality rates [22]. In 2012, an outbreak of *A. sobrai*–*A. veronii* complex was reported in Chinese soft-shelled turtles (*Pelodiscus maackii*) from several turtle farms in China, which caused severe economic losses for the turtle culturing industry [4].

However, healthy turtles can be asymptomatic carriers for the pathogen. Although motile *Aeromonas* spp. can cause extra-

*Correspondence to: Heo, G.-J., Laboratory of Aquatic Animal Medicine, College of Veterinary Medicine, Chungbuk National University, Chungdae-ro 1, Seowon-gu, Cheongju, Chungbuk 28644, Korea. e-mail: gjheo@cbu.ac.kr

#These authors contributed equally to this work.

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intestinal infections, such as septicemia, wound, soft tissue and skin infections in humans with preexisting diseases; they are mainly diarrheagenic pathogens causing gastroenteritis [17]. Although the pathogenesis of *Aeromonas*-induced gastroenteritis is complex and multifactorial, potential virulence factors have been reported and include cytotoxic heat-labile enterotoxin (*act*), cytotoxic heat-labile enterotoxin (*alt*) and heat-stable enterotoxin (*ast*). Furthermore, these virulence factors have been widely used in determining the potential pathogenicity of *Aeromonas* species [12, 15, 21, 27, 31].

The main obstacle against antimicrobial treatment of bacterial disease is the development of multiple antimicrobial resistances. Progressively increasing resistance to these agents is thus a serious cause of concern, and periodic monitoring of drug resistance of these organisms should be carried out in different geographical areas. Using such knowledge, the appropriate agents can be chosen for empiric therapy, as the emerging antimicrobial resistance of pathogenic bacteria worldwide is a compounding factor for the effective management of bacterial infections [26]. Recently, an increase in antimicrobial resistance of the genus *Aeromonas* has been reported [23, 29].

To assess the potential risk of indoor pet turtles as a carrier of *Aeromonas* species associated with infectious gastroenteritis causing diarrhea in humans, this study aimed to investigate virulence traits involving diarrhea and antimicrobial resistance pattern in *Aeromonas* species isolated from pet turtles and their environments.

MATERIALS AND METHODS

Purchase of pet turtles

Forty-two turtles of ten commercially popular species were purchased through pet shops and online markets in Korea. The randomly purchased turtles had an average weight of 15 ± 2 g, carapace diameter of 40 ± 5 mm and were under 4 weeks of age. All turtles were healthy and did not have any clinical signs of disease. Among the 42 turtles, 9 Chinese stripe-necked turtles (*Ocadia sinensis*), 5 yellow belly sliders (*Trachemys scripta scripta*), 11 river cooters (*Pseudemys concinna concinna*), 2 northern Chinese softshell turtles (*Pelodiscus maackii*), 3 western painted turtles (*Chrysemys picta belli*), 3 peninsula cooters (*Pseudemys peninsularis*), 2 African sideneck turtles (*Pelusios castaneus*), 3 common musk turtles (*Sternotherus odoratus*), 2 red belly cooters (*Pseudemys rubriventris*) and 2 alligator snapping turtles (*Macrolemys Temminckii*) were studied.

Raising condition of pet turtles

Eleven cages each containing two to nine turtles of the same species from the same pet shop were managed; each cage contained a slope made from soil and pebbles, 2l of sterilized water, and a canister filter to maintain water quality. The turtles were raised following the general husbandry method [3]: Gammarus dried shrimp with calcium supplements (Samhotech Co., Ltd., Seoul, Korea) were fed twice a day, while water temperature was kept within $26 \pm 2^\circ\text{C}$, pH 6.5–8.2 and 12 hr of photoperiod each day were maintained during the experiment.

Sample collection and bacterial isolation

Within one day of purchase, a fecal sample was taken from each turtle, and skin samples were collected by swabbing skin on the turtles' plastron with a sterilized cotton swab. After one week, five grams of soil from a turtle's rest or feeding area were obtained using a sterile spoon. Ten ml of water was collected from each cage with a sterile pipette. One ml of the suspension of each sample was inoculated into alkaline peptone water [19] and incubated at 37°C for 24 hr before streaking onto *Aeromonas* selective agar (Kisan Biotech Co., Ltd., Seoul, Korea). One hundred and two presumptive colonies were isolated based upon colony morphology on *Aeromonas* selective agar and Gram staining. These suspicious colonies were confirmed according to the phenotypic identification scheme [1].

Identification using gyrB gene sequence

After incubation of *Aeromonas* isolates, AccuPrep[®] genomic extraction kit (Bioneer, Daejeon, Korea) was used to purify bacterial DNA in the bacterial suspension. The concentration of purified DNA was determined using a Take3 session with a spectrophotometer (Epoch, Biotek, Wakefield, MA, U.S.A.) at 260, 280 and 320 nm. The species-level identification of 102 strains was performed with partial *gyrB* gene sequencing. The primer sequences, PCR amplification and sequencing were performed in accordance with previous reports [16, 28, 30]. The amplicons were purified using Accupower[®] gel purification kit (Bioneer) and sequenced at Cosmogenetech Co., Ltd. (Seoul, Korea). The partial *gyrB* sequence of each strain was separately blasted using the nucleotide collection (nr/nt) database (NCBI U.S.A.).

Detection of virulence genes

All strains were subjected to PCR assays to detect the three virulence genes (*act*, *alt* and *ast*) using the same primers and conditions described by Nawaz *et al.* [20] (Table 1). The generated amplicons were randomly selected and sequenced to demonstrate the specificity of each PCR assay.

Antimicrobial susceptibility test

The identified aeromonads were subjected to disk diffusion testing with nineteen common antibiotics. The testing was carried out according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards [7]. *Aeromonas* species were cultured on tryptic soy agar (MBcell Ltd., Seoul, Korea), and generated colonies were adjusted to a turbidity of McFarland

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

Name of gene	Primer sequence	Product size (bp)	Length (bp)	T _m (°C)
<i>act</i>	F: AGAAGGTGACCACCACCAAGAACA	232	24	65
	R: AACTGACATCGGCCTTGAAGCTC		24	
<i>alt</i>	F: TGACCCAGTCCTGGCACGGC	442	20	64
	R: GGTGATCGATCACCACCAGC		20	
<i>ast</i>	F: TCTCCATGCTTCCCTTCCACT	331	21	63
	R: GTGTAGGGATTGAAGAAGCCG		21	

Table 2. Distribution of *Aeromonas* species isolated from pet turtles and their environment based on *gyrB* sequencing

Turtle name	Species	Source (No. of isolates)
African sideneck turtle	<i>A. enteropelogenes</i>	Soil (1)
	<i>A. hydrophila</i>	Skin (2), feces (2) and water (1)
Alligator snapping turtle	<i>A. dhakensis</i>	Skin (1)
	<i>A. enteropelogenes</i>	Skin (1), soil (1) and water (1)
	<i>A. veronii</i>	Feces (2)
Chinese stripe-necked turtle	<i>A. caviae</i>	Feces (2)
	<i>A. dhakensis</i>	Skin (1) and feces (1)
	<i>A. enteropelogenes</i>	Skin (5), feces (1) and water (1)
	<i>A. hydrophila</i>	Skin (4), feces (3) and soil (1)
	<i>A. veronii</i>	Feces (1)
Common musk turtle	<i>A. enteropelogenes</i>	Skin (3), soil (1) and water (1)
	<i>A. hydrophila</i>	Feces (3)
Northern Chinese softshell turtle	<i>A. enteropelogenes</i>	Water (1)
	<i>A. hydrophila</i>	Skin (2), feces (2) and soil (1)
Peninsula cooter	<i>A. enteropelogenes</i>	Soil (1) and water (1)
	<i>A. hydrophila</i>	Skin (2) and feces (3)
	<i>A. veronii</i>	Skin (1)
Red belly cooter	<i>A. enteropelogenes</i>	Skin (1), feces (1) and soil (1)
	<i>A. hydrophila</i>	Skin (1) and feces (1)
River cooter	<i>A. caviae</i>	Feces (1)
	<i>A. dhakensis</i>	Feces (3)
	<i>A. enteropelogenes</i>	Skin (7), feces (4), water (1) and soil (1)
	<i>A. hydrophila</i>	Skin (1), feces (3) and soil (1)
	<i>A. veronii</i>	Water (1)
Western painted turtle	<i>A. enteropelogenes</i>	Skin (3), feces (3), water (1) and soil (1)
Yellow belly slider	<i>A. caviae</i>	Feces (1)
	<i>A. enteropelogenes</i>	Skin (5), feces (4), water (1) and soil (1)

0.5 (5×10^5 CFU ml⁻¹) with saline. The bacterial suspension was then spread on Mueller-Hinton agar (MBcell Ltd., Seoul, Korea). Amoxicillin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), nalidixic acid (30 µg) and tetracycline (30 µg) disks were prepared by independently soaking each paper disk with the given quantity of antibiotic. Disks containing amikacin (30 µg), aztreonam (30 µg), cefoxitin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), imipenem (10 µg), meropenem (10 µg), norfloxacin (10 µg), streptomycin (10 µg), tobramycin (10 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg) were purchased from Kisan Biotech Co., Ltd. (Seoul, Korea) and Oxoid Co., Ltd. (Seoul, Korea). Four to six disks were placed per inoculated MHA plate, and the plates were incubated for 24 hr at 30°C. After incubation, organisms were classified as susceptible (S), intermediately resistant (I) or resistant (R) on the basis of the size of the zone of bacteria growth inhibition according to the guidelines of the Clinical Laboratory Standards Institute [5].

RESULTS

Bacterial identification

One hundred and two isolates were identified based on biochemical analysis and partial *gyrB* gene sequencing as five *Aeromonas* species: 54 strains of *A. enteropelogenes* (52.9%), 33 strains of *A. hydrophila* (32.4%), 6 strains of *A. dhakensis* (5.9%), 5 strains of *A. veronii* (4.9%) and 4 strains of *A. caviae* (3.9%) (Table 2).

Table 3. Prevalence of *Aeromonas* strains having certain combinations of virulence genes

Gene combinations	Number (%) of positive strains for analyzed combination					Total positive strains
	<i>A. caviae</i> (n=4)	<i>A. dharkensis</i> (n=6)	<i>A. enteropelogenes</i> (n=54)	<i>A. hydrophila</i> (n=33)	<i>A. veronii</i> (n=5)	
<i>act</i> ⁺ / <i>alt</i> ⁻ / <i>ast</i> ⁻	-	-	1 (1.9)	-	5 (100)	6 (5.9)
<i>act</i> ⁻ / <i>alt</i> ⁺ / <i>ast</i> ⁻	-	5 (83.3)	-	-	-	5 (4.9)
<i>act</i> ⁻ / <i>alt</i> ⁻ / <i>ast</i> ⁺	1 (25.0)	-	2 (3.7)	-	-	3 (2.9)
<i>act</i> ⁻ / <i>alt</i> ⁺ / <i>ast</i> ⁺	-	-	-	1 (3.0)	-	1 (1.0)
<i>act</i> ⁺ / <i>alt</i> ⁻ / <i>ast</i> ⁺	-	-	-	7 (21.2)	-	7 (6.9)
<i>act</i> ⁺ / <i>alt</i> ⁺ / <i>ast</i> ⁺	-	-	-	25 (75.8)	-	25 (24.5)
Total	1 (25.0)	5 (83.3)	3 (5.6)	33 (100)	5 (100)	47 (46.1)

Table 4. Distribution of susceptible (S), intermediate (I) and resistance (R) strains of each *Aeromonas* species isolated from turtle and their environment

Antimicrobial agents	<i>A. caviae</i> (n=4)			<i>A. dharkensis</i> (n=6)			<i>A. enteropelogenes</i> (n=54)			<i>A. hydrophila</i> (n=33)			<i>A. veronii</i> (n=5)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Penicillins															
Amoxicillin (10 µg)	0	0	4	0	0	6	33	0	21	1	0	32	0	0	5
Ampicillin (10 µg)	0	0	4	0	0	6	32	0	22	2	0	31	0	0	5
Cephalosporins															
Cephalothin (30 µg)	0	0	4	0	0	6	0	0	54	15	0	18	1	0	4
Ceftriaxone (30 µg)	3	0	1	2	1	3	52	0	2	26	3	4	4	1	0
Cefoxitin (30 µg)	2	0	2	2	0	4	46	3	5	27	3	3	4	0	1
Cefotaxime (30 µg)	4	0	0	1	0	5	49	5	0	33	0	0	5	0	0
Carbapenems															
Imipenem (10 µg)	3	0	1	6	0	0	54	0	0	32	0	1	5	0	0
Meropenem (10 µg)	4	0	0	6	0	0	54	0	0	33	0	0	5	0	0
Aminoglycosides															
Amikacin (30 µg)	3	0	1	6	0	0	54	0	0	33	0	0	5	0	0
Streptomycin (10 µg)	1	1	2	4	0	2	47	6	1	31	1	1	4	0	1
Gentamycin (10 µg)	2	1	1	6	0	0	54	0	0	33	0	0	5	0	0
Tobramycin (10 µg)	2	0	2	6	0	0	54	0	0	33	0	0	5	0	0
Fluoroquinolone															
Ciprofloxacin (5 µg)	2	0	2	2	2	2	13	30	11	32	0	1	5	0	0
Norfloxacin (10 µg)	2	0	2	0	0	6	10	25	19	33	0	0	3	0	2
Others															
Aztreonam (30 µg)	4	0	0	6	0	0	47	1	6	32	1	0	5	0	0
Chloramphenicol (30 µg)	0	0	4	0	0	6	5	0	49	7	0	26	0	0	5
Nalidixic acid (30 µg)	3	0	1	4	0	2	44	6	4	24	3	6	3	1	1
Tetracycline (30 µg)	3	0	1	2	0	4	4	0	50	15	1	17	2	0	3
Trimethoprim / sulfamethoxazole (1.25/23.75 µg)	2	0	2	0	0	6	44	6	4	26	6	1	4	0	1

S, susceptible; I, intermediate; R, resistance.

Detection of virulence genes

In PCR assays for detecting enterotoxin-encoding genes (Table 3), 53.9% (n=55) of the present isolates did not harbor any enterotoxin genes. One or more enterotoxin genes were detected in 46.1% (n=47) of isolates. Twenty-five out of 47 strains were tested positive for all enterotoxin genes. Based on the combinations of enterotoxin genes detected in PCR assays, the *Aeromonas* spp. could be divided into the following seven genotypes: *act*⁺/*alt*⁻/*ast*⁻, *act*⁻/*alt*⁻/*ast*⁺, *act*⁺/*alt*⁺/*ast*⁺, *act*⁻/*alt*⁺/*ast*⁺, *act*⁻/*alt*⁺/*ast*⁻, *act*⁺/*alt*⁻/*ast*⁺ and *act*⁻/*alt*⁻/*ast*⁻. The *act*⁻/*alt*⁻/*ast*⁻ genotype was observed in 3 isolates of *A. caviae*, 1 isolate of *A. dhakensis* and 51 isolates of *A. enteropelogenes*. Five out of six *A. dhakensis* isolates possessed only the *alt* gene (*act*⁻/*alt*⁺/*ast*⁻ genotype). All *A. veronii* isolates were *act*⁺/*alt*⁻/*ast*⁻ genotype. On the other hand, all *A. hydrophila* isolates possessed two or more enterotoxin genes. In addition, 25 out of 33 *A. hydrophila* isolates tested positive for all enterotoxin genes in PCR assays.

Antimicrobial susceptibility test

Table 4 shows the antimicrobial susceptibilities of the present *Aeromonas* spp. using a disk diffusion test. Most of the present isolates were susceptible to all antibiotics except amoxicillin, ampicillin, cephalothin, chloramphenicol and tetracycline. The

β -lactams belonging to the penicillin class were resisted by 66.7% of the present isolates. *A. enteropelogenes* isolates exhibited lower antibiotic resistant rates to amoxicillin and ampicillin, but higher resistance to ciprofloxacin compared to other *Aeromonas* spp. However, resistance to aztreonam was shown only in six *A. enteropelogenes* isolates. These isolates showed antimicrobial resistant rates of over 90% to cephalothin, chloramphenicol and tetracycline. Resistances to aminoglycosides and cephalosporins were frequently observed among *A. caviae* and *A. dhakensis* isolates, respectively. In the case of *A. caviae*, one isolate was resistant to imipenem.

DISCUSSION

Pet turtles are well-known carriers of pathogenic *Salmonella* spp. causing human salmonellosis. Because of the risk of infection transferred by physical contact, the FDA has banned the sale of young turtles with a shell length of less than 4 inches size in the US [8]. Besides *Salmonella* spp., healthy turtles could be carriers of many opportunistic pathogens for humans, such as *Aeromonas* species. *Aeromonas* spp. are widely distributed in aquatic environments and have frequently been isolated in healthy and diseased aquatic animals including turtles. However, there is little information available about the potential pathogenicity and antimicrobial resistance of *Aeromonas* spp. carried by aquatic pet turtles.

A. enteropelogenes, now considered a synonym of *A. trola*, has rarely been isolated from healthy animals including humans and aquatic environments [11]. *A. hydrophila*, *A. caviae* and *A. veronii* were commonly found in environmental and clinical sources worldwide, among which *A. hydrophila* was the predominant species in the intestinal tract of aquatic animals [24]. In the case of *A. dhakensis*, it has been isolated from a variety of sources since its first description but rarely in turtles [22]. In contrast to previous studies, the present study showed that *A. enteropelogenes* ($n=54$) was the predominant species among isolates recovered from the feces and skin of pet turtles and their rearing environments followed by *A. hydrophila*. We also identified *A. caviae* ($n=4$), *A. veronii* ($n=5$) and *A. dhakensis* ($n=6$) from a minority of turtles. Based on these results, *A. enteropelogenes* and *A. hydrophila* were considered to be normal flora of the pet turtles used for the present study.

A. caviae, *A. veronii*, *A. dhakensis* and *A. hydrophila* have been recognized as important pathogens within the genus *Aeromonas* [12]. They were previously reported to cause intestinal, extraintestinal and wound infections in a variety of host animals. *A. enteropelogenes* has frequently been isolated from diarrheal patients and is associated with gastroenteritis [25]. *Aeromonas* enterotoxin genes (*act*, *alt* and *ast*) have been used for investigating the potential enterotoxicity of isolates recovered from clinical, food and environment samples [21]. In light of this, the present isolates were subjected to PCR assays using primer sets specific to these enterotoxin genes for evaluation of their potential pathogenicity. Most *A. enteropelogenes* isolates (51/54) did not harbor any enterotoxin genes (*act*⁻/*alt*⁻/*ast*⁻ genotype). In contrast, all *A. hydrophila* isolates harbored more than one enterotoxin gene, among which 25 isolates possessed the *act*⁺/*alt*⁺/*ast*⁺ genotype. A previous study using animal models showed that *Aeromonas* spp. harboring more than two enterotoxin genes caused more severe diarrhea than *Aeromonas* spp. with a single enterotoxin gene [27]. In addition, there are genotypic differences based on the combination of enterotoxin genes between clinical and non-clinical *Aeromonas* isolates [2]. Based on the present and previous studies, *A. hydrophila* with all enterotoxin genes could pose a serious threat to public health. We therefore suggest that pet turtles are a significant reservoir for enterotoxigenic *Aeromonas* infection in humans.

Although recent studies show an emergence of non-susceptible isolates of *Aeromonas* to a variety of antibiotics [23], the majority of *Aeromonas* isolates are generally susceptible to most antimicrobial agents except for penicillins. However, *A. enteropelogenes* (also known as *A. trola*) has been reported to be susceptible to penicillins, but resistant to cephalothin and cefazolin. According to De Luca *et al.* [6], *A. enteropelogenes* showed a unique pattern of susceptibility to ampicillin and resistance to cephalothin arising from the production of a single inducible cephalosporinase. In contrast to this study, the present study showed a frequent occurrence of resistant isolates to penicillins among *A. enteropelogenes* isolates with resistance to cephalothin. Therefore, a further study is needed to determine whether these isolates of *A. enteropelogenes* can produce inducible β -lactamases, such as penicillinase and cephalosporinase. On the other hand, the present isolates showed high resistance to tetracycline and chloramphenicol. Similar findings have frequently been reported from previous studies conducted on antimicrobial susceptibility of *Aeromonas* species in Asia [29]. In addition, 76% of the present *A. enteropelogenes* isolates were non-susceptible to ciprofloxacin. The prevalence was higher in the present study than in previous studies. In agreement with previous studies, a minority of the present isolates were resistant to imipenem, amikacin, ceftriaxone and cefotaxime. As imipenem has been reported to be highly successful for treating *Aeromonas* infections [13], resistance to this antimicrobial in particular is highly significant. The emergence of these resistant isolates from pet turtles may pose significant threat to public health, as their resistance pattern could prevent treatment of human infections.

A. enteropelogenes was the predominant species among *Aeromonas* spp. isolated from pet turtles followed by *A. hydrophila*. In addition, the present study revealed an unusual antimicrobial resistance pattern among *A. enteropelogenes* isolates and high frequency of *A. hydrophila* with potential enterotoxicity. Collectively, these results indicate that healthy pet turtles might serve as a potential reservoir for enterotoxigenic *Aeromonas* infection that prove difficult to treat with antimicrobial agents.

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