THE PRESENCE OF PUTATIVE VIRULENCE DETERMINANTS, TETRACYCLINE AND β-LACTAMS RESISTANCE GENES OF Aeromonas SPECIES ISOLATED FROM PET TURTLES AND THEIR ENVIRONMENT

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Abstract: This study aimed to characterize Aeromonas spp. isolated from ten popular species of pet turtles and their environment to evaluate the potential risk of pet turtles as a source of virulence-associated genes, and tetracycline and β-lactams resistance determinants. Presence of eight virulence genes (ser, aer, exu, lip, fla, ascV, ahyB and gcat), and tetracycline (tetA, tetB and tetE) and β-lactams (blaTEM, blaSHV, blaOXA and blaCTX-M) resistance genes were evaluated by conventional PCR assays. The aerA gene showed the highest frequency of occurrence (92%), followed by fla (75%), gcaT (68%), ahyB (59%), ser (39%), lip (37%) and ascV (25%) genes. None of the isolates carried amplicon of DNase-associated exu gene. A. hydrophila, A. dharkensis, A. veronii and A. caviae were carried seven tested virulence genes except for exu while only four virulence genes were detected in A. enteropelogenes. Among the 75 tetracycline-resistant isolates, tetA, tetE and tetB genes were detected in 38, 26 and 6 isolates, respectively. Among the tested β-lactam resistance genes, blaOXA and blaTEM genes were detected in 54% and 36% of β-lactam resistant isolates, respectively. No blaCTX-M and blaSHV genes were detected. Our results indicate that pet turtle-associated aeromonads, exhibiting potential virulence and antimicrobial (tetracycline and β-lactams) resistance genes, may pose a serious health risk to pet turtle owners, particularly to immunocompromised individuals.

Key words: Aeromonas spp.; virulence-associated genes; tetracycline resistance; β-lactams resistance; pet turtle

Introduction

Mesophilic aeromonads are ubiquitous bacteria that are a component of the normal microbiota of many aquatic animals such as fish, amphibians, and reptiles (1). They can cause ulcerative stomatitis, pneumonia, dermatitis, and septicemia in reptiles under stressful conditions such as trapping, handling and temperature variations of rearing environment (2, 3). Over the years, many studies have been investigated to evaluate the prevalence of Aeromonas species in aquatic animals, mainly food-producing animals (4, 5). However, a limited number of studies evaluating the distribution of aeromonads in pet turtles have been published up to date (6, 7). The pathogenesis of Aeromonas species involves various virulence factors including cytotoxic heat-labile enterotoxin (act), cytotoxic heat-labile enterotoxin (alt) and cytotoxic heat-stable enterotoxin (asf), aerolysin (aer), lipase (lip), serine protease (ser), elastase (ahyB), DNase (exu), glycerophospholipid-cholesterol acyltransferase (gcaT), flagellar system (fla) and Type III secretion system (TTSS) effector (ascV). These genes encoding virulence factors have been broadly used in determining the potential pathogenicity of Aeromonas species isolated from the environment, foodstuffs, and human clinical samples (1, 8-10).
Recently, antibiotic-resistant aeromonads have been recognized as a serious concern due to their potential health risks to animals and humans (11, 12). Especially, the dissemination of tetracycline and β-lactams resistance aeromonads in the aquatic environment has been widely documented (12, 13, 14). Among many tetracycline resistance genes, the tetE, tetA and tetB genes were frequently identified from Aeromonas species in the aquatic environment (12, 15, 16). Aeromonas species can produce numerous β-lactamases for conferring resistance to β-lactams. According to isolation sources, the previous studies have shown the different prevalence of genes encoding β-lactamases in Aeromonas species. In the aquatic environment, the blaxygen, blaOXA, and blaCTX-M β-lactams genes were frequently detected from Aeromonas species (17-19).

These resistance genes containing plasmids and transposons are known as mobile genetic elements that can be transferred horizontally among distantly related lineages. Particularly, The aquatic environment is more favorable for the transmission of resistant bacteria, thus, Aeromonas species as opportunistic pathogens might be dangerous vectors for the spreading of antibiotic resistance genes through the aquatic environment (18, 20). Hence, the present study was conducted to determine the occurrence of antimicrobial resistance genes (tetracyclines and β-lactams) and virulence-associated genes of Aeromonas species isolated from pet turtles and their rearing environment.

Materials and methods

Bacterial isolates

One hundred and two Aeromonas species isolates obtained from ten commercially popular pet turtles species (Chinese stripe-necked turtles Ocadia sinensis, yellow belly sliders Trachemys scripta scripta, river cooters Pseudemys concinna concinna, northern Chinese softshell turtles Pelodiscus maackii, western painted turtles Chrysemys picta belli, peninsula cooters Pseudemys peninsularis, African sideneck turtles Pelusios castaneus, common musk turtles Sternotherus odoratus, red belly cooters Pseudemys rubriventris and alligator snapping turtles Macroclemys temminckii) and their rearing environment was screened to investigate the presence of putative virulence, and β-lactams and tetracycline resistance genes. These isolates have been previously characterized for their antimicrobial susceptibilities, enterotoxin (act, alt and ast) genes and quinolone resistance determinants (7, 21).

Detection of antibiotic resistance genes

Twenty-eight and seventy-five isolates were selected (21) for the detection of β-lactams and tetracycline resistance determinants, respectively. These isolates were tested by PCR assays to detect the genetic determinants associated with resistance to β-lactams (blaTEM, blaSHV, blaOXA and blaCTX-M), and tetracyclines (tetA, tetB and tetE). The primer sets used in PCR amplification are summarized in table 1. PCR amplifications were conducted in 20 μL volumes consisting of 10 μL of Quick Taq® HS DyeMix (Toyobo, Japan), 1 μL of 10 pmol/μL each primer and 1 μL of the template under standard conditions. The PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels. Positive controls were implemented with previously characterized enterobacterial strains that harbored the corresponding genes (21, 22).

Detection of virulence-associated genes

All isolates were subjected to PCR assays to detect the 8 tested virulence genes including ser, aer, exu, lip, fla, ascV, ahyB and gcat. The PCR amplification of the virulence-associated genes was carried out according to the PCR primers and conditions reported previously (Table 1). The PCR mixture of 20 μL contained 10 μL Quick Taq HS DyeMix (Toyobo, Japan), 7 μL PCR water, 1 μL template and 1 μL of each primer. The PCR products were examined by electrophoresis on 1.5% (W/V) agarose gel.

Results

Bacterial isolates

One hundred and two Aeromonas species isolates 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%) 7.
Presence of resistance genes

Among the tested β-lactam resistance genes, \( \text{bla}_{\text{OXA}} \) and \( \text{bla}_{\text{TEM}} \) genes were detected in 54% and 36% of β-lactam resistant isolates, respectively. No \( \text{bla}_{\text{CTX-M}} \) and \( \text{bla}_{\text{SHV}} \) genes were detected (Table 2). Among the 75 tetracycline-resistant isolates, \( \text{tetA} \), \( \text{tetE} \) and \( \text{tetB} \) genes were detected in 38, 26 and 6 isolates, respectively (Table 3).

Table 1: Oligonucleotide primers and PCR conditions \(^a\) used to amplify virulence and antibiotic resistance genes of *Aeromonas* spp.

| Gene | Target | Nucleotide Sequence (5'-3') | Size (bp) | Annealing temperature (°C) | Reference |
|------|--------|-----------------------------|-----------|---------------------------|-----------|
| \( \text{aerA} \) | Aerolysin | F: CTATGGCCCTGAGCCAGGAAG R: CAGTTCAGTCACCACCT | 431 | 62 | 30 |
| \( \text{ser} \) | Serine protease | F: ACCGGAAGTATGGGTAGCAAGGC R: GCTCATGCCGTAACCTGGTT | 350 | 55 | 13 |
| \( \text{fla} \) | Flagella | F: CCAACCGTCTGCTTCCTCCAC G: MYTGGTGGCGGCGTGTT | 608 | 36 | |
| \( \text{ahyB} \) | Elastase | F: CACCTTCTCCAGCTGGTTCGG R: CCGTGCCAGGGACGCTGGTTT | 382 | 36 | |
| \( \text{lip} \) | Lipase | F: ATCTTCTCCAGCCATGGTTCGG R: CCGTGCCAGGGACGCTGGTTT | 382 | 36 | |
| \( \text{exu} \) | DNase | F: AGACATGCACAACTCTTCC G: GATTGGTATTGCCTTGCGAG | 323 | 59 | 13 |
| \( \text{gcaT} \) | Glycerophospholipid-cholesterol acyltransferase | F: TCCTGGAATCCCAAGTATCAG G: GCAGGTTGAACAGCAGTATCT | 237 | 65 | 13 |
| \( \text{ascV} \) | Type III Secretion System | F: AGCAGATGATGATCGTGAAGGC G: GAGGATTCTCCATGGTACCAG | 891 | 58 | 38 |
| \( \text{bla}_{\text{TEM}} \) | \( \beta \)-lactams resistance | F: ATAAAATCTCTGAGCGAGAAA G: GAAGCTATGAAATGTCGAATC | 1080 | 60 | |
| \( \text{bla}_{\text{SHV}} \) | \( \beta \)-lactams resistance | F: TTATCTCCCTGGATAGCCACC G: GATTGCTGATTTGCTGGTG | 795 | 60 | |
| \( \text{bla}_{\text{CTX-M}} \) | \( \beta \)-lactams resistance | F: CGCTTCTCCAGTGTCAGG G: ACCGCCTATCCGTTGGT | 550 | 52 | 22 |
| \( \text{tetA} \) | Tetracycline resistance | F: GTAACTTCTGAGACCTGTCG R: CCGCAGTACTGGTGGCTAG | 1000 | 62 | |
| \( \text{tetB} \) | Tetracycline resistance | F: CTCAGTATCCCAAGCCTTTT G: CTAAGCATTCTCCTCTT | 400 | 57 | 22 |
| \( \text{tetE} \) | Tetracycline resistance | F: GTGATGATGACCTGGTCATR: CTGTCGTTACATGTCGTT | 1100 | 62 | |

\(^a\) PCR thermocycle conditions for each reaction; initial denaturation of 94 °C for 2 min followed by a total of 35 cycles of amplification. Each cycle consisted of 94 °C denaturation for 30 s, annealing for 50 s and 72 °C extension for 10 min.
### Table 2: β-lactams resistance profiles of turtle-associated *Aeromonas* spp.

| Isolate | Host a | β-lactam resistance b | β-lactam resistance genes |
|---------|--------|------------------------|---------------------------|
| **Aeromonas caviae** | | | |
| AD14 | CSN | AMP, AMX, CEP, FOX | bla<sub>TEM</sub> |
| AC50 | RC | AMP, AMX, CEP, FOX, IMI | bla<sub>OXA</sub>, bla<sub>TEM</sub> |
| **A. dharkensis** | | | |
| AD17 | RC | AMP, AMX, CEP, CRO, FOX, CTX | bla<sub>OXA</sub>, bla<sub>TEM</sub> |
| AD18 | RC | AMP, AMX, CEP, CRO, FOX, CTX | bla<sub>OXA</sub>, bla<sub>TEM</sub> |
| AD19 | RC | AMP, AMX, CEP, FOX | bla<sub>OXA</sub> |
| AD15 | CSN | AMP, AMX, CEP, FOX, CTX | bla<sub>CDA</sub> |
| **A. enteropelogenes** | | | |
| AC2 | RC | AMP, AMX, CEP | bla<sub>OXA</sub> |
| AC6 | RC | CEP, FOX | - |
| AC15 | NCS | AMP, AMX, CEP, FOX | bla<sub>OXA</sub> |
| AC30 | CM | CEP, CTX, ATM | - |
| AC31 | WP | CEP, CTX, ATM | - |
| AC32 | WP | CEP, CTX, ATM | - |
| AC35 | RC | CEP, CTX, ATM | - |
| AC44 | YB | AMP, AMX, CRO | bla<sub>TEM</sub> |
| AC45 | RC | CEP, CRO, ATM | - |
| AC53 | WP | AMP, AMX, CEP, FOX | bla<sub>OXA</sub> |
| AV4 | RC | AMP, AMX, CEP, FOX | bla<sub>CDA</sub> |
| AD1 | CM | AMP, AMX, CEP, FOX | bla<sub>CDA</sub> |
| **A. hydrophila** | | | |
| AH1 | RC | AMP, CEP | - |
| AH11 | CSN | AMP, AMX, CEP, CRO | bla<sub>OXA</sub>, bla<sub>TEM</sub> |
| AH13 | CSN | CEP, CRO, FOX, IMI | - |
| AH19 | NCS | AMP, AMX, CEP, CRO | bla<sub>OXA</sub> |
| AH20 | NCS | AMP, AMX, CEP | bla<sub>TEM</sub> |
| AH22 | YB | AMP, AMX, CEP | - |
| AH23 | YB | AMP, AMX, CEP, CRO | bla<sub>OXA</sub> |
| AH25 | CM | AMP, AMX, CEP, FOX | bla<sub>TEM</sub> |
| AD10 | AF | AMP, AMX, CEP, FOX | bla<sub>OXA</sub>, bla<sub>TEM</sub> |
| **A. veronii** | | | |
| AC52 | SN | AMP, AMX, CEP, FOX | bla<sub>OXA</sub>, bla<sub>TEM</sub> |

a Host: CSN= Chinese stripe-necked turtle, YB= yellow belly slider, RC= river cooter, PC= peninsula cooter, NCS= northern Chinese softshell turtle, CM= common musk turtle, WP= western painted turtle, AF= African sideneck turtle, SN= Alligator snapping turtle.
b β-lactams resistance: AMX=Amoxicillin (10 µg), AMP=Ampicillin (10 µg), CEP=Cephalothin (30 µg), CRO=Ceftriaxone (30 µg), FOX=Cefoxitin (30 µg), CTX=Cefotaxime (30 µg), IMI=Imipenem (10 µg)

### Table 3: Distribution of tetracycline resistance genes among tetracycline resistant *Aeromonas* species isolated from pet turtles and their environment

| Species | Number of positive isolates (Subtotal %) |
|---------|-----------------------------------------|
|         | tetA | tetB | tetE |
| Aeromonas enteropelogenes (n = 50) | 32 (64) | - | 8 (2) |
| A. hydrophila (n = 17) | 6 (35) | - | 12 (71) |
| A. dharkensis (n = 4) | - | 2 (50) | 3 (75) |
| A. veronii (n = 3) | - | 3 (100) | 2 (66) |
| A. caviae (n = 3) | - | 1 (100) | 1 (100) |
| Total (%) (n = 75) | 38 (51) | 6 (1) | 26 (35) |
Distribution of virulence-associated genes

The occurrence and frequencies of virulence genes are shown in Table 4. The aerA gene showed the highest frequency of occurrence (92%), followed by fla (75%), gcaT (68%), ahyB (59%), ser (39%), lip (37%) and ascV (25%) genes. None of the isolates carried amplicon of the DNase-associated exu gene.

Discussion

The Aeromonas spp. under study were multidrug-resistant turtle-associated bacteria which carried quinolone resistance determinants, as well as enterotoxin genes (7, 21). The isolates were highly resistant to β-lactams especially amoxicillin, ampicillin and cephalothin. β-lactam antibiotics have been used for the treatment of Aeromonas infection during the last decade. However, their efficacy has significantly declined due to the production of β-lactamases by resistant bacterial strains (14, 17, 23). The Aeromonas spp. are naturally resistant to β-lactams because of the expression of chromosomal β-lactamases (24).

In this study, twenty-eight aeromonads isolates were resistant to the more than one β-lactam antibiotics. Among them, 54% and 36% of isolates harbored blaOXA and blaTEM genes. Several previous studies have documented the detection of the blaOXA and blaTEM genes in Aeromonas isolates recovered from the environment (14, 25) and clinical samples (26) and the prevalence of gene detection varies according to the isolation sources. In Korea, a previous study reported that the blaOXA and blaTEM genes were detected in 3% and 100% of Aeromonas isolates from aquaculture fish (14). However, a different trend was observed in this study which the blaOXA and blaTEM genes were detected in Aeromonas isolates from pet turtles that suggest a wide distribution of β-lactamase genes in Aeromonas isolates from various sources.

A much higher level of tetracycline resistance was observed amongst aeromonads in our previous study (7) and 78 of tetracycline-resistant isolates were selected to detect their tetracycline resistance determinants (tetA, tetB and tetE). A. enteropelogenes and A. hydrophila harbored tetA and tetE genes while other Aeromonas species harbored tetB and tetE genes. Previous reports indicate that the tetA and tetE determinants are the predominant tetracycline resistance genes in the aquatic environment (16, 27) and both genes code for an efflux pump that eliminates the drug from the cell (28). The tetA, tetB and tetE genes are located on the plasmid as well as tetA in the transposon (Tn1721) and tetE is adjacent to the integrons (15). Han et al. (27) has reported that tetE gene was the predominant tetracycline determinant in Aeromonas spp. isolated from Korean fish farms and aquariums. However, Kim et al. (29) reported that tetA was the most frequent gene in A. salmonicida strains isolated from salmonid farms and private aquariums in Korea. The tetB gene was detected at a low frequency, while Jacobs and Chenia. (12) reported a lower prevalence of tetB genes among Aeromonas spp. isolated from the South African aquaculture system.

Detection of virulence encoding genes of Aeromonas spp. have been widely applied for evaluating their potential pathogenicity (30, 31). However, the prevalence of virulence-associated genes has rarely been reported in Aeromonas strains from pet turtles (7). In the current study, Aeromonas isolates were found to possess genes aerA, lip, ahyB, ser, fla, gcaT and ascV, while genes for DNase (exu) was not identified. Especially, none of A. enteropelogenes isolates harbored lip, ser, exu and ascV genes.
genes. Previous studies have revealed that multiple virulence-associated genes are present in *Aeromonas* isolates and having high heterogeneity in the distribution of virulence-associated genes (10, 30, 31). The pore-forming aerolysin/hemolysin encoded *aer* gene was the most prevalent in this study which was detected in 92% of the total isolates representing all species of the genus. Several studies have reported the high prevalence of the *aer* gene in clinical and environmental *Aeromonas* isolates (30, 32).

The three enterotoxins *act*, *alt*, and *ast* have been implicated as major virulence factors in diarrheal disease which had been investigated in our previous study (7). However, the presence of these toxins might not be enough for virulence (31). The temperature-stable metalloprotease with elastolytic activity (*ahyB*) and serine protease (*ser*) play an important role in the invasiveness and establishment of infection (1). In the current study, the *ahyB* and *ser* genes were detected in 59% and 39% of isolates, respectively. None of the *A. enteropelogenes* isolates harbored *ser* gene. The flagella are important appendages for the initial attachment of bacteria to the gastrointestinal epithelium and involve in the subsequent adherence process and biofilm formation (33, 34). The *fla* gene-encoded polar flagella were common among the *Aeromonas* isolates from the aquatic environment. The *fla* gene was detected in 99% of *Aeromonas* isolates from diseased eel in Korea (10).

The *gcaT* gene plays a coherent, integrated role in the establishment of pathogenicity of *Aeromonas* spp. by involving in the regulation and secretion of extracellular glycerophospholipid-cholesterol acyltransferase (13). The *gcaT* gene was detected in 68% of *Aeromonas* isolates.

Lipases play a role as virulence factors by interacting with leukocytes or by disturbing several immune system functions through free fatty acids produced by the lipolytic activity. Extracellular lipases secreted by *Aeromonas* spp. actively involve in the alteration of the host plasma membrane and thus increase the severity of infection (35). Among *Aeromonas* strains isolated in the present study, 91% of *A. hydrophila*, 60% of *A. veronii*, 50% of *A. dharkensis* and 50% of *A. caviae* isolates were found to have *lip* gene. Several previous studies reported a high prevalence of *lip* gene among the *Aeromonas* isolates from the aquatic environment (10, 36). Type III secretion system (T3SS) plays a crucial role in host-pathogen interactions by injecting effector toxins directly into the cytosol of host cells (37). The *ascV* gene encodes the T3SS and which was detected in 59% of *Aeromonas* spp. except for *A. enteropelogenes* isolates. The presence of *ascV* gene was previously detected in 68% of *Aeromonas* spp. isolated from diseased farmed fish and farm environment (38). Besides, the high frequency of *ascV* gene was reported in human clinical isolates (37).

The *exu* gene is responsible for DNA hydrolysis which was not detected in this study. The absence of *exu* gene was also reported by Nawaz et al. (13) in *A. veronii* isolated from catfish in the USA. In contrast, the high prevalence of *exu* gene was observed in *Aeromonas* spp. isolated from freshwater lakes in Malaysia (39) and diseased eel in South Korea (10).

The specificity of the host or environmental source could be the possible reasons for the absence of *exu* gene in this study.

According to the available literature, this is the first description of these virulence-associated genes in *Aeromonas* of pet turtle origin. Most of *Aeromonas* strains isolated from pet turtles and their environment harboring multiple virulence-associated genes have the potential to be pathogenic. Turtle born aeromonads carrying tetracycline and β-lactams resistance determinants can disseminate through the environment. Collectively, which may pose a public health risk to pet turtle owners, particularly to immunocompromised individuals.

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