Detection and distribution of virulence genes in *Aeromonas hydrophila* isolates causing infection in cultured carps

Mina Ahangarzadeh*, Masoud Ghorbanpour Najafabadi†, Rahim Peyghan‡, Hossein Houshmand¶, Mostafa Sharif Rohani*, Mehdi Soltani*§

1 South of Iran Aquaculture Research Institute, Iranian Fisheries Science Research Institute, Agricultural Research Education and Extension Organization (AREEO), Ahvaz, Iran; 2 Department of Pathobiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran; 3 Department of Aquatic Animal Health, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran; 4 Department of Fish diseases, Iranian Fisheries Research Science Institute (IFSR), Agriculture Research, Education and Extension Organization (AREEO), Tehran, Iran; 5 Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; 6 Centre for Sustainable Aquatic Ecosystems, Harry Butler Institute, Murdoch University, Perth, Australia.

**Abstract**

*Aeromonas hydrophila* is a bacterium associated with many diseases and disorders such as fin rot, skin ulcers and lethal hemorrhagic septicemia in fish. It bears several virulence factors including type III secretion system (T3SS), aerolysin, cytolytic enterotoxin and enzymes (e.g., hemolysins, lipase) that seem to play an important role in its pathogenesis. Detection of virulence markers by polymerase chain reaction (PCR) is a key procedure in defining the pathogenic ability of pathogenic bacteria and preparing a vaccine for its treatment. In this sense, this study was aimed to determine the frequency of virulence genes in isolates obtained from infected cultured carps in Khuzestan province. Out of 200 moribund carps with septicemic symptoms, 125 isolates were belonged to the motile aeromonads and 59 isolates were identified as *A. hydrophila* by biochemical methods. Finally, using PCR analysis, 31 isolates were identified as *A. hydrophila*. Five virulence genes were detected in these isolates including hemolysin, aerolysin, cytolytic enterotoxin and T3SS (aopB and ascV) by specific primers. Results showed that 23 (74.19%), 18 (58.06%), 16 (51.61%), 13 (41.63%) and 10 (32.25%) isolates possessed cytolytic enterotoxin, hemolysin, aerolysin, and T3SS genes, respectively. The results of the present study showed that among 31 isolates, only five isolates had all of dominant virulence genes. Thirteen other isolates had genotypes including hlyA, aerA, and act. The remaining isolates had at least one virulence gene. This study showed that determination of the virulence genes by PCR can be a reliable method to identify a potential pathogenic *Aeromonas* strain.

© 2022 Urmia University. All rights reserved.

**Introduction**

The pathogenic bacteria are the most important factors that affect general health of farmed fish.1 *Aeromonas hydrophila* is a straight, coccobacillary Gram-negative bacterium with rounded ends.2 This motile facultative anaerobe bacterium is an important pathogen causing primary or secondary infectious disease in warm water farmed fish. The stress condition such as temperature fluctuations, handling or poor water quality have critical role in Aeromonad infections in fish.3 The pathogenesis of *A. hydrophila* infection is multi-factorial and associated with a number of virulence factors including cell structural lipopolysaccharides, outer-membrane proteins, pili and flagella, type III secretion system (T3SS), and extracellular factors such as enzymes and toxins. The exotoxins such as aerolysin (aerA), cytolytic enterotoxin (act), and hemolysin (hlyA) as well as enzymes such as lipase, proteases, and nuclease seem to play an important role in the pathogenesis.4,7 The virulence genes of *A. hydrophila* are generally divided into three main groups of hemolysin, aerolysin, cytolytic enterotoxins.8 The inactivation of either hemolysin or aerolysin genes reduced the hemolytic and cytotoxic activities of virulent strains of *A. hydrophila*, however, it could not totally inactivate these endotoxins.9 The type III secretion system (T3SS) in Gram-negative

---

*Correspondence:
Mina Ahangarzadeh, DVM, PhD
South of Iran Aquaculture Research Institute, Iranian Fisheries Science Research Institute, Agricultural Research Education and Extension Organization (AREEO), Ahvaz, Iran.
E-mail: m.ahangarzadeh@areeo.ac.ir

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License which allows users to read, copy, distribute and make derivative works for non-commercial purposes from the material, as long as the author of the original work is cited properly.
bacteria such as *Aeromonas salmonicida* and different isolates of *A. hydrophila* may result in their virulence. AscV and aopB genes are important components of T:SS in *A. hydrophila* that encode the T:SS consolidation proteins associated in internal membrane of bacteria and make holes or channels in the host cell and have been used for the present survey of T:SS in screening tests of bacterial strains.  

The detection method of virulence genes was recently proposed as a reliable approach to identify a potential pathogenic Aeromonas strain. In recent years several molecular methods, particularly polymerase chain-reaction (PCR) based method have been developed for routine identification of Aeromonas species and their putative virulence genes. The present study was aimed to detect selected virulence genes (hemolysin, aerolysin, cytolytic enterotoxin, and T:SS) in isolates obtained from diseased cultured carps in Khuzestan province, Iran.

**Materials and Methods.** A total number of 200 moribund cultured carps with septicemic symptoms including: hemorrhage, ascites, exophthalmos, lethargy, visceral adhesions, and liver discoloration were collected from 30 carp culture farms in Khuzestan province, Iran. Then samples were taken from internal organs including head kidney, liver, spleen, and intestine and also skin lesions. They were streaked on tryptic soy agar (TSA; Merck, Darmstadt, Germany) and incubated at 37.00 °C for 24 hr. Following incubation, typical colonies (entire circular, convex, white to greyish, semi-translucent with size of 2.00 to 3.00 mm) were selected from each plate with a pure culture and streaked onto new TSA plates.

**Phenotypic identification.** Gram-negative isolates positive for oxidase and catalase were considered to be suspected for *A. hydrophila*. Then isolates were classified as *A. hydrophila* according to their reactions in the following conventional tests: Motility, arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase, indole, Voges-Proskauer, citrate (Simmon's), starch, urea, ascelin, arabinose, cellobiose, sucrose, and salicin. Each substrate was incubated at 37.00 °C and reactions were read after 24 and 48 hr.  

**Bacterial DNA extraction for PCR.** The genomic DNA was extracted by boiling method. In this regard, each isolate was grown at 37.00 °C overnight on tryptic soy broth (TSB) medium. Then they were centrifuged (5,000 g for 10 min). The pellets were re-suspended in 1.00 mL phosphate buffer saline (PBS; pH 7.50) in tubes followed by centrifugation (5,000 g for 10 min). The pellets were re-suspended in 250 μL of diethyl pyrocarbonate (DEPC) water (Sigma, St. Louis, USA), boiled for 10 min and then centrifuged (5,000 g for 10 min). Finally, 200 μL of the cell lysates were stored at –20.00 °C until the PCR analysis.

The identification of *A. hydrophila* and its virulence genes with PCR. Polymerase chain-reaction was performed with the primers targeted to 16S rRNA and lipase genes in *A. hydrophila*. A final volume of 25.00 μL of PCR mixture containing 2.00 μL of template DNA and 12.50 μL of a master mix (containing each deoxy-nucleoside triphosphate, MgCl2, and Taq DNA polymerase), 1.00 μL of a 10.00 mM per μL of each primer and 8.50 μL of sterile double distilled water was used for assay with a Thermal Cycler (Corbett Life Science, Sydney, Australia). Each reaction was considered as negative (for negative controls, DNA preparation was replaced by PCR water) and positive controls to confirm the accuracy of the reaction. Then, the PCR products were analyzed by electrophoresis on 1.50% agarose gel with safe stain and visualized by UV trans-illumination. If the 685 bp band was observed for genes 16S rRNA and 763 bp for the lipase gene, the isolate was considered as *A. hydrophila*. In addition, five virulence genes including hemolysin (hlyA), aerolysin (aerA), cytolytic (act) enterotoxin, aopB and ascV) in *A. hydrophila* were amplified by PCR with the same procedures with some modifications for providing the optimal condition (Table 1).

**Table 1. Cycling conditions in PCR used for each primer of genes.**

| Genes                  | Cycles | Temperature-Time Cycle No. |
|------------------------|--------|---------------------------|
| Initial denaturation    | 94.00 ˚C - 5 min | 1                      |
| Denaturation            | 94.00 ˚C - 30 sec | 30                     |
| Annealing               | 65.00 ˚C - 30 sec | 30                     |
| Extension               | 72.00 ˚C - 45 sec | 30                     |
| Final extension         | 72.00 ˚C - 5 min | 30                     |
| **16S rRNA**            |        |                           |
| Initial denaturation    | 94.00 ˚C - 5 min | 1                      |
| Denaturation            | 94.00 ˚C - 30 sec | 30                     |
| Annealing               | 68.00 ˚C - 30 sec | 30                     |
| Extension               | 72.00 ˚C - 2 min | 30                     |
| Final extension         | 72.00 ˚C - 5 min | 30                     |
| **Lipase**              |        |                           |
| Initial denaturation    | 94.00 ˚C - 5 min | 1                      |
| Denaturation            | 94.00 ˚C - 30 sec | 30                     |
| Annealing               | 55.00 ˚C - 30 sec | 30                     |
| Extension               | 72.00 ˚C - 30 sec | 30                     |
| Final extension         | 72.00 ˚C - 5 min | 30                     |
| **Hemolysin (hlyA)**    |        |                           |
| Initial denaturation    | 94.00 ˚C - 5 min | 1                      |
| Denaturation            | 94.00 ˚C - 30 sec | 30                     |
| Annealing               | 58.00 ˚C - 30 sec | 30                     |
| Extension               | 72.00 ˚C - 30 sec | 30                     |
| Final extension         | 72.00 ˚C - 5 min | 30                     |
| **Aerolysin (aerA)**    |        |                           |
| Initial denaturation    | 94.00 ˚C - 5 min | 1                      |
| Denaturation            | 95.00 ˚C - 1 min | 30                     |
| Annealing               | 58.50 ˚C - 50 sec | 30                     |
| Extension               | 72.00 ˚C - 1 min | 35                     |
| Final extension         | 72.00 ˚C - 5 min | 1                      |
| **Cytolytic enterotoxin (act)** | | |
| Initial denaturation    | 95.00 ˚C - 5 min | 1                      |
| Denaturation            | 95.00 ˚C - 1 min | 30                     |
| Annealing               | 58.50 ˚C - 50 sec | 30                     |
| Extension               | 72.00 ˚C - 1 min | 35                     |
| Final extension         | 72.00 ˚C - 5 min | 1                      |
| **AscV - AopB**         |        |                           |
The primers were designed according to the sequences deposited in GenBank database and references (Table 2).\textsuperscript{19,22-24} The size of the expected PCR products for virulence genes in the 1.50% agarose gel were 597, 252, 482, 137 and 129 bp for hlyA, aerA, act, AscV and AopB genes, respectively.

### Results

**Isolation and Identification of A. hydrophila by biochemical and PCR.** Out of 200 samples collected form fish with septicemic signs, 125 isolates were belonged to the motile Aeromonads of which 59 isolates were identified as A. hydrophila by biochemical methods (Table 3). Out of 59 isolates, 31 isolates were identified as A. hydrophila by PCR analysis (Fig. 1).

Table 2. Primers used for the amplification of virulent genes of Aeromonas hydrophila.

| Virulent genes          | Primers | DNA sequences (5’-3’) | Product size (bp) | References |
|-------------------------|---------|----------------------|-------------------|------------|
| Hemolysin(hlyA)         | F       | GGC CGG TGG CCC GAA GAT GCA GG | 597               | 23         |
|                         | R       | GGCC GGC GGA GGA GAC GGG |       |            |
| Aerolysin (aerA)        | F       | GCA GAA CCC ATC TAT CCA G | 252               | 20         |
|                         | R       | TTT CTC CGG TAA CAG GAT TG |       |            |
| Cytolytic enterotoxin (act) | F   | ATG ACC CAG TGC TGG CAC GG | 482               | 24         |
|                         | R       | GCC GCT CAG GGC GAA GCC GC |       |            |
| AscV                    | F       | GGCAGAATGTTGTTGGCGTTT | 137               | 25         |
|                         | R       | AACATCGTGCTGATTCTGGA |       |            |
| AopB                    | F       | TCCAGCAAGTTCGCTGTTT | 129               | 25         |
|                         | R       | GCFCATGAAGGCCTAAAT |       |            |

### Table 3. Biochemical identification of Aeromonas hydrophila. Data are presented as percentage.

| Biochemical tests         | Buller\textsuperscript{18} | Characteristics of isolates identified as A. hydrophila by both PCR and biochemical tests |
|---------------------------|-----------------------------|--------------------------------------------------------------------------------------------|
| Positive                  | Negative                    |                                             | Positive | Negative |
| Gram stain                | -                           | 100                                         |          |          |
| Motility                  | +                           | -                                           |          |          |
| Oxidase                   | +                           | 100                                         |          | -        |
| Catalase                  | +                           | 100                                         |          | -        |
| Indole                    | +                           | 100                                         |          | -        |
| H2S                       | -                           | -                                           |          | 100      |
| Arginine decarboxylase    | +                           | 100                                         |          | -        |
| Lysine decarboxylase      | +                           | 80.64                                       | 19.36    |          |
| Ornithine decarboxylase   | -                           | 87.09                                       | 12.90    |          |
| Urea                      | -                           | -                                           |          | 100      |
| Simmon's citrate          | +(60.00%)                   | 83.87                                       | 16.13    |          |
| Voges-Proskauer           | +                           | 64.51                                       | 35.49    |          |
| Nitrate production        | +                           | 100                                         |          | -        |
| Aesculin                  | +                           | 93.55                                       | 6.45     |          |
| Acid from Arabinose       | variable                    | 48.39                                       | 51.61    |          |
| Mannitol                  | +                           | 100                                         |          | -        |
| Sucrose                   | +                           | 93.55                                       | 6.45     |          |
| Sorbitol                  | -                           | 83.87                                       | 16.13    |          |
| Inositol                  | -                           | 19.36                                       | 80.64    |          |
| Lactose                   | -                           | 12.90                                       | 87.10    |          |
| NaCl (0-3)                | +                           | 100                                         |          | -        |
Presence of virulence factors in genomic DNA of \textit{A. hydrophila} isolates. PCR assay showed that 18 (58.60%), 16 (51.61%), 23 (74.19%), 13 (41.63%), 10 (32.25%) of the 31 isolated strains were positive for \textit{hlyA}, \textit{aerA}, \textit{act}, \textit{ascV} and \textit{aopB}, respectively (Fig. 2). Based on the detection of virulence genes two dominant genotypes in \textit{A. hydrophila} isolates were found. The \textit{hlyA*}, \textit{aerA*} and \textit{act*} genotypes were the most common genotype in 13 (41.93%) isolated strains. In addition, \textit{hlyA*}, \textit{aerA*}, \textit{act*}, \textit{aopB*} and \textit{ascV*} genotype was found in 5 (16.12%) of all of isolated strains (Table 4).

Table 4. Virulence properties of \textit{Aeromonas hydrophila} isolates.

| Isolates No. | \textit{hlyA} | \textit{aerA} | \textit{act} | \textit{ascV} | \textit{aopB} |
|--------------|---------------|---------------|--------------|--------------|--------------|
| 1            | +             | +             | +            | +            | +            |
| 2            | +             | +             | +            | -            | -            |
| 3            | +             | +             | +            | +            | +            |
| 4            | +             | +             | +            | +            | -            |
| 5            | -             | -             | +            | +            | +            |
| 6            | +             | +             | +            | -            | -            |
| 7            | +             | -             | +            | +            | -            |
| 8            | +             | -             | -            | -            | -            |
| 9            | +             | +             | +            | -            | -            |
| 10           | -             | +             | +            | -            | -            |
| 11           | +             | +             | +            | +            | +            |
| 12           | -             | -             | +            | -            | -            |
| 13           | +             | +             | +            | -            | -            |
| 14           | +             | +             | +            | +            | +            |
| 15           | +             | +             | +            | +            | +            |
| 16           | -             | -             | +            | -            | -            |
| 17           | +             | -             | +            | -            | -            |
| 18           | +             | +             | +            | -            | -            |
| 19           | +             | +             | +            | -            | -            |
| 20           | +             | -             | +            | -            | -            |
| 21           | +             | +             | +            | -            | -            |
| 22           | +             | +             | +            | -            | -            |
| 23           | -             | -             | -            | -            | -            |
| 24           | -             | -             | -            | -            | -            |
| 25           | -             | -             | +            | -            | -            |
| 26           | -             | -             | +            | +            | +            |
| 27           | -             | +             | +            | -            | -            |
| 28           | -             | -             | -            | -            | -            |
| 29           | -             | -             | +            | -            | -            |
| 30           | -             | -             | -            | -            | -            |
| 31           | +             | +             | +            | +            | +            |

Fig. 2. Detection of A) hemolysin, B) aerolysin, C) act D) ascV and E) aopB genes in \textit{A. hydrophila} on 1.50% agarose gel. A) Lanes 1: Ladder (100 bp), 2: Control negative, 3, 4 and 5: PCR amplification of hemolysin gene (597 bp), 6: Control positive; B) Lanes 1: Ladder (100 bp), 2: Control negative, 3, 4 and 5 and 6: PCR amplification of aerolysin gene (252bp), 7: Control positive; C) Lanes 1: Control negative, 2, 3 and 4: PCR amplification of aerolysin gene (482 bp), 5: Control positive, 6: Ladder (100bp); D) Lanes 1: PCR amplification of ascV gene (137 bp), 2: Control positive, 3: Ladder (100 bp); E) Lanes 1: Control negative, 2 and 3: PCR amplification of aopB gene (129 bp), 4: Control positive, 5: Ladder (100 bp).

The activity of these toxins were prohibited when both \textit{hlyA} and \textit{aerA} genes were suppressed.\textsuperscript{26} \textit{Aerolysin} is an extracellular, soluble and hydrophilic protein exhibiting both hemolytic and cytolytic properties. \textit{Aerolysin} binds to specific glycoprotein receptors on the surface of eukaryotic cells before inserting into the lipid bilayer and forming holes. Hemolysins are exotoxin proteins produced by bacteria and the lytic activities of hemolysins on red blood cells are reported.\textsuperscript{27} Some researchers reported that the screening of virulence determinants was a reliable method to identify a potential pathogenic \textit{Aeromonas} strain by using PCR.\textsuperscript{4,6,22,27}

In the present study, the percentage of occurrences of \textit{hlyA} and \textit{aerA} genes were 58.06 and 51.61, respectively using specific primers in \textit{Aeromonas} isolates. Also, in 41.93% of responsible isolates in \textit{Aeromonas} septicemia both genes were observed. In similar studies, different frequency of occurrence from these genes was recorded in \textit{A. hydrophila} isolates.\textsuperscript{4,28} The cause of differences could be due to temporal and spatial variations of studied isolates and the used primer.

Yogananth et al. showed that 50.00% of the isolated organism had both \textit{hly} and \textit{aer} genes, which to some extent was similar to the present study.\textsuperscript{4} Yousr et al. have investigated \textit{hly} and \textit{aer} genes in \textit{Aeromonas} isolates and showed 52.63% of isolates were PCR positive for these genes.\textsuperscript{28} Ye et al. indicated that 85.00% of \textit{A. hydrophila}...
isolates from cultured carp with hemorrhagic septicemia were positive for the presence of hly and aer genes that had higher frequency compared to the findings of our study. Moreover, it has been found that 100% of the isolated A. hydrophila from infected tilapia had hly and aer genes. Uma et al. demonstrated the presence of hly and aer genes in A. hydrophila isolates obtained from infected Koi carp and they were presented as virulent strain.

In this study, the frequency of cytotoxic enterotoxin genes was estimated 74.19%. Twenty-three strains of 31 isolates amplified the sequence with 482 bp, which indicates the presence of cytotoxic enterotoxin gene. Furthermore, it has been revealed that 35.00% of A. hydrophila isolates from cultured carp with hemorrhagic septicemia were cytotoxic enterotoxin gene positive. The cause of differences could be due to temporal and spatial variations of studied isolates and the used primers.

Type III secretory is an effective system for transportation of toxin into the target cells (effector proteins), and plays an important role in interactions between pathogen and host, and is known as one of the major virulence factors of Gram negative bacteria in plant and animal species. Several studies have shown the importance of this system by inactivation or removing some coding genes in bacterial virulence and pathogenicity. For example, Yu et al. showed that inactivation of aopB and aopD genes of T3SS in A. hydrophila delayed cytotoxicity on carp epithelial cells and reduced its virulence in Gourami fish. Also, the presence of T3SS in wild species of the Pseudomonas aeruginosa compared to a mutant of this bacteria (the secretory system was disabled) was shown to be more virulent and caused higher mortality in infected animals. The results of these research demonstrated the importance and role of the T3SS in the pathogenesis of many Gram-negative bacteria, especially Aeromonas sp. Based on the results of the present study five strains were shown to have five dominant genes, hlyA+, aerA+ act+ aopB+ ascV+ genotypes and 13 isolates had hlyA+ aerA+ act+ genotypes. The results showed that nine isolates had both genes of T3SS. Finally, it was shown that the most frequent genotypes were hlyA+ and act+.

Aslani et al. worked on human diarrheal cases and reported that most genotypes among isolates were belonged to hlyA+ aerA+ genotype with an abundance of 78.6 percent. The difference between the results of previous studies with current study might be due to variation in the origin of the isolates of A. hydrophila.

Finally, traditional methods for the detection of the virulence properties in Aeromonas sp. are based on biological assays in vitro and in vivo, using cell lines and animal models, respectively. However, these only reveal the phenotypic characteristics of the strains, while the expression of the putative virulence-associated factors in Aeromonas appears to be affected by environmental conditions.

For this reason, these methods could in some instances fail to indicate the potential pathogenicity of isolates. Screening of specific cytotoxin and hemolysin genes appears to be the most effective way of detecting and characterizing Aeromonas virulence factors.

Acknowledgment

The authors thank the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran and South of Iran Aquaculture Research Institute, Ahvaz, Iran.

Conflict of interest

The authors declare there is no conflict of interests.

References

1. Zorrilla I, Chapillard M, Arijo S, et al. Bacteria recovered from diseased cultured gilthead sea bream ( Sparus aurata L.) in southwestern Spain. Aquaculture 2003; 218: 11-20.
2. Al-Fatlawy HNK, Al-Ammar MH. Molecular study of Aeromonas hydrophila isolated from stool samples in Najaf (Iraq). Int J Microbiol Res 2013; 5(1): 362-365.
3. Inglis V, Roberts RJ, Bromage NR. Bacterial diseases of fish. New York, USA: Halsted Press 1993: 312.
4. Yogananth N, Bhakayaraj R, Chanthuru A, et al. Detection of virulence gene in Aeromonas hydrophila isolated from fish samples using PCR technique. J Biotechnol Biochem 2009; 4(1): 51-53.
5. Janda JM, Abbott SL, The genus Aeromonas: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 2010; 23(1): 35-73.
6. Albert MJ, Ansaruzzaman M, Talukder KA, et al. Prevalence of enterotoxin genes in Aeromonas spp. isolated from children with diarrhea, healthy controls, and the environment. J Clin Microbiol 2000; 38(10): 3785-3790.
7. Heuzenroeder MW, Wong CY, Flower RL. Distribution of two hemolytic toxin genes in clinical and environmental isolates of Aeromonas spp.: correlation with virulence in a suckling mouse model. FEMS Microbiol Lett 1999; 174(1): 131-136.
8. Kingombe CI, Huys G, Tonolla M, et al. PCR detection, characterization, and distribution of virulence genes in Aeromonas spp. Appl Environ Microbiol 1999; 65(12): 5293-5302.
9. Wong CY, Mayrhofer G, Heuzenroeder MW, et al. Measurement of virulence of aeromonads using a suckling mouse model of infection. FEMS Immunol Med Microbiol 1996; 15(4): 233-241.
10. Burr SE, Stuber K, Wahl T, et al. Evidence for a type III secretion system in Aeromonas salmonicida subsp. salmonicida. J Bacteriol 2002; 184(21): 5966-5970.
11. Francis MS, Wolf-Watz H, Forsberg A. Regulation of type III secretion systems. Curr Opin Microbiol 2002; 5(2): 166-172.
12. Dean P. Functional domains and motifs of bacterial type III effector proteins and their roles in infection. FEMS Microbiol Rev 2011; 35(6): 1100-1125.
13. Chacón MR, Soler L, Groisman EA, et al. Type III secretion system genes in clinical Aeromonas isolates. J Clin Microbiol 2004; 42(3): 1285-1287.
14. Sen K. Development of a rapid identification method for Aeromonas species by multiplex-PCR. Can J Microbiol 2005; 51(11): 957-966.
15. Chang Y-C, Wang J-Y, Selvam A, et al. Multiplex PCR detection of enterotoxin genes in Aeromonas spp. from suspect food samples in northern Taiwan. J Food Prot 2008; 71(10): 2094-2099.
16. Nielsen ME, Høi L, Schmidt AS, et al. Is Aeromonas hydrophila the dominant motile Aeromonas species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China? Dis Aquat Organ 2001; 46(1): 23-29.
17. Buller NB. Bacteria and fungi from fish and other aquatic animals: a practical identification manual. 2nd ed. Oxford, UK: CABI 2014; 137-277.
18. Ottaviani D, Parlini C, Citterio B, et al. Putative virulence properties of Aeromonas strains isolated from food, environmental and clinical sources in Italy: a comparative study. Int J Food Microbiol 2011; 144(3): 538-545.
19. Porteen K, Agarwal RK, Bhilegaonkar KN. PCR-based detection of Aeromonas from milk samples. J Food Technol 2006; 4(2): 111-115.
20. Dorsch M, Ashbolt NJ, Cox PT, et al. Rapid identification of Aeromonas species using 16S rDNA targeted oligonucleotide primers: a molecular approach based on screening of environmental isolates. J Appl Bacteriol 1994; 77(6): 722-726.
21. Cascón A, Anguita J, Hernanz C, et al. Identification of Aeromonas hydrophila hybridization group 1 by PCR assays. Appl Environ Microbiol 1996; 62(4): 1167-1170.
22. Aslani MM, Seyyed Hamzeh H. Characterization and distribution of virulence factors in Aeromonas hydrophila strains isolated from fecal samples of diarrheal and asymptomatic healthy persons, in Ilam, Iran. Iran Biomed J 2004; 8(4): 199-203.
23. Granum PE, O’Sullivan K, Tomás JM, et al. Possible virulence factors of Aeromonas spp. from food and water. FEMS Immunol Med Microbiol 1998; 21(2): 131-137.
24. Shirali T. Effect of para coumaric acid on expression of some virulence genes of Aeromonas hydrophila and its protective effects in Comman Carp (Cyprinus carpio). PhD Thesis. Shahid Chamran University of Ahvaz, Ahvaz, Iran: 2015.
25. Lee S, Kim S, Oh Y, et al. Characterization of Aeromonas hydrophila isolated from rainbow trouts in Korea. J Microbiol 2000; 38(1): 1-7.
26. Wong CYF, Heuzenroeder MW, Flower RLP. Inactivation of two haemolytic toxin genes in Aeromonas hydrophila attenuates virulence in a suckling mouse model. Microbiology (Reading) 1998; 144(Pt 2): 291-298.
27. Uma A, Rebecca G, Meena S, et al. PCR detection of putative aerolysin and hemolysin genes in an Aeromonas hydrophila isolate from infected Koi carp (Cyprinus carpio). Tamil Nadu J Vet Anim Sci 2010; 6(1): 31-33.
28. Your Ah Napis S, Rusul GRA, et al. Detection of aerolysin and hemolysin genes in Aeromonas spp. isolated from environmental and shellfish sources by polymerase chain reaction. ASEAN Food J 2007; 14(2): 115-122.
29. Ye YW, Fan TF, Li H, et al. Characterization of Aeromonas hydrophila from hemorrhagic diseased freshwater fishes in Anhui Province, China. Int Food Res J 2013; 20(3): 1449-1452.
30. Castro-Escarpulli G, Figueras MJ, Aguiler-Aurreola G, et al. Characterisation of Aeromonas spp. isolated from frozen fish intended for human consumption in Mexico. Int J Food Microbiol 2003; 84(1): 41-49.
31. Tomás JM. The main Aeromonas pathogenic factors. ISRN Microbiology 2012; 2012: 256261. doi: 10.5402/2012/256261.
32. Yu HB, Rao PSS, Lee HC, et al. A type III secretion system is required for Aeromonas hydrophila AH-1 pathogenesis. Infect Immun 2004; 72(3): 1248-1256.
33. Vance RE, Rietsch A, Mekalanos JJ. Role of the type III secreted exoenzymes S, T, and Y in systemic spread of Pseudomonas aeruginosa PA01 in vivo. Infect Immun 2005; 73(3): 1706-1713.
34. Schuelt G, Feltman H, Rabin SDP, et al. Secretion of the toxin ExoU is a marker for highly virulent Pseudomonas aeruginosa isolates obtained from patients with hospital-acquired pneumonia. J Infect Dis 2003; 188(11): 1695-1706.
35. Merino S, Aguilar A, Rubires X, et al. Mesophilic Aeromonas strains from different serogroups: the influence of growth temperature and osmolarity on lipopolysaccharide and virulence. Res Microbiol 1998; 149(6): 407-416.