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Virulence Determinants and Plasmid Profile of *Aeromonas hydrophila* Strains Isolated from *Oreochromis niloticus*

A.M. Younes, A.Y. Gaafar and Elham S. Awad

Hydrobiology Department, National Research Centre (NRC),
El-Bhooth Street (Formerly El-Tahrir St.) Dokki, Giza 12622, Egypt

Abstract: This study shed light on distribution of virulence determinants and plasmids profile of *Aeromonas hydrophila* strains from diseased fish. A total of 11 phenotypically identified *A. hydrophila* isolates recovered from *Oreochromis niloticus* suffering from septicemia in Egypt were genetically characterized by sequencing of 16srRNA. All isolates were screened by PCR for 6 virulence determinant genes (act, ast, alt, lip, ela, fla). All isolates contained one or multiple genes, with a heterogeneous distribution and Act and Lip being the most prevalent (81.8%). Isolates were analyzed for the presence of small plasmids. The results showed that one isolate harbored three plasmids of different size.

Key words: *Aeromonas hydrophila* · Nile tilapia · *Oreochromis niloticus* · Virulence genes · Plasmids

INTRODUCTION

Aeromonas species are ubiquitous in aquatic ecosystems and are frequently found in foods, including meat,fish, vegetables, fresh and seawater. *Aeromonas* spp. can infect wide hosts such as fish, domestic chicken, lower and higher vertebrates and human [1, 2]. *A. hydrophila* is the causative agent of motile aeromonad septicemia (MAS) in fish causing high mortality in wild, farmed freshwater and marine fish species affecting the economics of the fish industry throughout the world. The symptoms of MAS include swelling of tissues, dropsy, red sores, necrosis, ulceration and hemorrhagic septicemia [3, 4].

The pathogenicity of *A. hydrophila* is usually considered to be multifactorial including cytotonic heat-labile(alt) and cytotonic heat-stable enterotoxins (ast), cytotoxic heat-labile enterotoxin (act), aero lysin (aer), flagella A and flagella B (fla), lipase (lip), elastase (ela), serine protease (ser), ADP ribosyl transferase toxin (aexT) and DNases (exu) secretion systems, quorum systems, iron acquisition and antibiotic resistance [2-5].

Presence of naturally occurring plasmids encoding different virulence factors has been identified in the genus *Aeromonas* [6, 7]. *A. hydrophila* strains bear plasmids considers of a potential public health hazard, because they may be transferred from animals to human [1, 8, 9].

Therefore, the objectives of this study are to perform biochemical and molecular identification for the presence of the virulence gene in the isolates. Also, to investigate the presence and number of plasmids bearing isolates.

MATERIALS AND METHODS

Samples and Biochemical Identification: A total of 11 strains of *A. hydrophila* were isolated from moribund *O. niloticus* at a private fish farm, Kafr El-Sheikh governorate, Egypt. The strains were isolated from the kidney of individual diseased fish and cultivated on *Aeromonas* selective agar base (LabM, UK) supplemented with Ampicillin. The suspected *Aeromonas* isolates were identified by oxidase, catalase and sensitivity to Vibrio-static reagent O/129 (Sigma). The isolates were then biochemically identified using API20NE strips (BioMerieux, France). The purified isolates of *A. hydrophila* were stored in Tryptic soya broth (TSB) with 20% (vol/vol) glycerol at -80°C.

Genomic DNA Isolation: Bacterial isolates were grown on TSA (Lab M, UK) and incubated at 25°C for 24 h. Bacterial colonies were pelleted then extraction of genomic DNA was carried out using DNA extraction kit (Fermentas, Lithuania) according to manufacturer's suggested protocol for Gram-negative bacteria. The purified bacterial

Corresponding Author: Alkhateib Y. Gaafar, Veterinary Research Division, National Research Centre, Cairo, Egypt.

Table 1: Oligonucleotides sequences of primers used in this study

Gene name	Nucleotide sequence	Size of product (bp)	References
16srRNA	63f 5- CAGGCCTAACACATGCAAGTC-3	1300	Marchesi *et al.* [10]
	1387r 5- GGGCGGWGTGTACAAGGC-3		
Act	F 5'-AGAAGGTGACCACCAAGAACA-3'	232	Sen and Rodgers, [2]
	R 5'-AACTGACATCGGCCTTGAACTC-3'		
Ast	F 5'-TCTCCATGCTTCCCTTCCACT-3'	331	
	R 5'-GTGTAGGGATTGAAGAAGCCG-3'		
Fla	F 5'-TCCAACCGTYTGACCTC-3'	608	
	R 5'-GMYTGGTTGCGRATGGT-3'		
Alt	F 5'-TGACCCAGTCCTGGCACGGC-3'	442	
	R 5'-GGTGATCGATCACCACCAGC-3'		
Lip	F 5'-ATCTTCTCCGACTGGTTCGG- 3'	382	
	R 5'-CCGTGCCAGGACTGGGTCTT-3'		
Ela	R 5'-ACACGGTCAAGGAGATCAAC-3'	513	
	R 5'-CGCTGGTGTTGGCCAGCAGG-3'		

DNA was eluted into 50 µL of elution buffer and subjected to spectrophotometric measurement. The primer pairs used for PCR amplification and sequencing of 16S rRNA and the specific conditions for investigating 16S rRNA were as reported previously [10]. The amplified fragment of 16S rRNA gene of *A. hydrophila* isolates was sequenced using Sanger DNA sequencer (Applied Biosystem) in two directions. BLAST in the NCBI was used for verifying the amplified products.

Detection of Virulence Genes: All *Aeromonas* strains were subjected to PCR assays to detect the presence of six virulence genes; cytotoxic heat-labile enterotoxin (Act), cytotonic heat-stable enterotoxins (Ast), Lipase (Lip), elastase (Ela), cytotonic heat-labile (Alt) and flagella A and flagella B (Fla) using the same primers sequences and PCR conditions described by Sen and Rodgers [2]. The sequence of each primer used to amplify the target genes, the expected size of the PCR products and their references are documented in Table (1).

Plasmid DNA Isolation: Plasmid miniprep kit (Thermo Scientific, USA) was used, as recommended by the manufacturer, to extract <50-kb plasmids from the isolates. The plasmid preparations were separated by regular gel electrophoresis (0.8%) at 90 V for 60 min. The gels were stained with ethidium bromide to visualize the DNA bands under Gel Documentation (BioRad).

RESULTS

Bacterial Isolation and Identification: All bacterial isolates were motile, Gram-negative, short bacilli, oxidase and catalase positive, resistant to Vibrio-static reagent O/129. Further biochemical identification of these species using the API20NE confirmed *A. hydrophila* with two different API20NE profile numbers.

Detection of Virulence Genes: There are several reports suggesting that motile *Aeromonas* isolates could carry either one or multiple virulence determent which play an important role in the pathogenesis. The results in this study showed the distribution of the six virulence genes within eleven *A. hydrophila* isolates. There was a variety in the distribution of virulence factors in the *A. hydrophila* isolates (Table 2). All isolates (100%) harbored one or more virulence gene. But No isolate was found to have all six genes. Four isolates (36.36%) had five virulence genes.

The prevalence of virulence genes of fish *Aeromonas* isolates were as follows: Act and Lip genes the most frequent virulence genes were detected in 9 (81.8%) strains. The sizes of the amplification products were identical with those predicted from the designed primers (Table 1). The PCR revealed the presence of an amplification product at 232bp and 382bpcharacteristic for the genus *Aeromonas* Act and Lip. The putative virulence gene Ela was detected in 7 (63.63%) isolates. Alt gene was detected in 6 (54.54%) isolates, while, Fla gene was detected in 5 (45.45%). Finally, Ast gene was detected in just one isolate (0.09%).

Plasmid DNA Isolation: In this study, the results of plasmids isolation from 11 *A. hydrophila* showed that, only one isolate had 3 bands representative for 3 plasmids of about ~2.7kb, ~5.8kb and ~14kb (Fig 1). Another isolated harbored one plasmid of about 14kb. Whereas, other isolates were negative to carry any plasmids.

Table 2: Distribution of six virulence genes within 11 *A. hydrophila* isolates obtained by PCR amplification

	Putative virulence genes (%)					
Total Number of isolates	ACT	AST	LIP	ELA	ALT	FLA
11	9(81.8 %)	1(0.09%)	9(81.8 %)	7(63.63%)	6(54.54%)	5(45.45%)

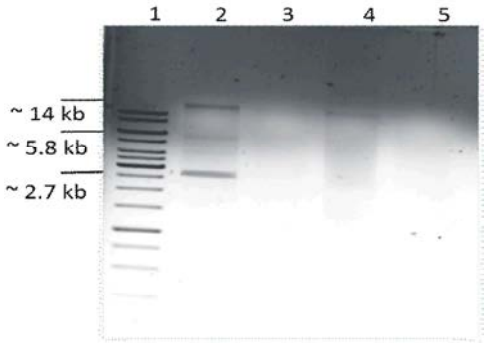

Fig. 1: Agarose gel electrophoresis of plasmid profile of some *A. hydrophila* isolates. Lane 1: kb molecular DNA marker. Lane 2: *A. hydrophila* isolate bearing 3 plasmids. Lane 4: *A. hydrophila* isolate bears one plasmid.

DISCUSSION

Aeromonas species are widespread in aquatic ecosystems, are frequently isolated from fresh and estuarine water, surface waters, sewage, healthy ordiseased fish, food products, animal and humanfeces and may cause infections in humans and fish [1, 3].

We identified the presence of six virulence genes from *A. hydrophila* strains isolated from diseased *O. niloticus* from private fish farms. The cytotoxic enterotoxin encoded by the act gene of *A. hydrophila*, has cytotoxic, hemolytic and enterotoxic activity. Therefore, we used Act primer as described by Sen and Rodgers [2] to amplify a 232-bp fragment for detecting the act/aerA/hlyA gene. The obtained results showed that Act was the most prevalent virulence gene (81.8%). This is in agreement with earlier studies thatthe occurrence of hemolytic factors in aeromonads is widespreadand their presence can be used as indicators of virulence [11]. This percent is higher than that previously reported by Kingombe *et al.* [12], who found 65% of the 350 clinical and environmental *Aeromonas* strains positive for act/hlyA/aerA, while Sen and Rodgers [2] found that, the major enterotoxin act/hlyA/aerA was present in 70% of the 205 *A. hydrophila* isolates from drinking water. Higher to our results, Nawaz *et al*. [5] detected rate of *aer*A gene as 96% in integrons in *A. veronii* isolated from catfish.

Similar to Act, the gene encoding Lip was the most prevalent and detected in 81.8% of the isolates. In agreement with our results Lip was detected in all *A. hydrophila* fish isolates from Egypt [13]. Lipase alters the structure of the cytoplasmic membrane of the host tissue cells causing their necrosis and helps the colonization of *A. hydrophila* [14]. The previous study reported that *A. hydrophila* with insertion mutants for the lipase gene reduced the lethal dose in mice and fish models [15].

The presence of lateral flagella and swarmer motility was a pathogenic factorin mesophilic *Aeromonas* isolates [16]. Flagella are important in the adherence process. It has been shown that mutations inthe polar flagellum flaA and flaB genes result in complete lossof motility and adherence to human epithelial HEp-2 cells [2]. In this study the Fla gene was detected in 5 (45.45%). In consistence with this result, Kirov *et al*. [17] detected the *fla* gene in about 50% of clinical, environmental and reference strains, while, Gavín *et al*. [16] detected the *fla* gene in 62% of clinical *Aeromonas* isolates and in 70% of fish isolates.

The Ast gene was detected in one (0.09%) isolate. Similar to this result Nawaz *et al*. [5] did not detect the *alt* and *ast* genes in fish isolates whereas, Sen and Rodgers [2] found that 30% of the *A. hydrophila* isolates had Ast gene.

Aeromonas spp. are known to carry plasmids encoding antibiotic resistance and/or virulence genes [6, 7]. Plasmids are considered the predominant factors mediating horizontal gene transfer and facilitate the exchange of genetic information between bacteria in the environment and between thehuman and aquaculture environments [9].

The results of plasmid profile showed that one isolate carry three small plasmids (~2.7, ~5.8 and ~14 kb). One isolate carry one plasmid (~14 kb). Other isolates bear no plasmids. There are studies which reported *Aeromonas* strains harboring one or more plasmids. Furmanek-Blaszk [18] isolated *A. hydrophila* strain from River Nile near Aswan Dam carried a small pAhy2.5 plasmid (2524 bp). Strains of *A. hydrophila* identified to carry a single plasmid of 21 kb in India [7]. Han *et al.* [19] discoverd in *A. sobria* and *A. hydrophila* smallplasmids (pAQ2-1 and

pAQ2-2) bearing a quinolone resistance gene (*qnrS2*). Three small cryptic plasmids pAsa1, pAsa2, pAsa3 (5.0, 5.2 and 5.4 kb respectively) were discovered in *A. salmonicida* subsp. *salmonicida*. pAsa1 and pAsa3 are ColE2-type replicons while pAsa2 is a ColE1-type replicon [6]. *A. salmonicida* isolates may be an important plasmid reservoir and bear large plasmids involved in bacterial virulence and drug resistance [9].

CONCLUSION

All *Aeromonas* strains isolated had one or multiple virulence factors and thus have the potential to be pathogenic. Treatment of these pathogenic bacteria could be difficult and expensive. Plasmids play an important role in antimicrobial multiple-drug resistance and virulence. Some isolates carry one or three plasmids of different size which mediate horizontal gene transfer, between bacteria in the environment and between the human, pose as public health threats. A detailed study of virulent plasmids would help in understanding the pathogenesis and effective control.

REFERENCES

1. Janda, J.M. and S.L. Abbott, 2010. The genus *Aeromonas*: taxonomy, pathogenicity and infection. Clinical Microbiology Review, 23: 35-73.
2. Sen, K. and M. Rodgers, 2004. Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. Journal of applied microbiology, 97(5): 1077-1086.
3. Beaz-Hidalgo, R. and M.J. Figueras, 2013. *Aeromonas* spp. whole genomes and virulence factors implicated in fish disease. Journal of Fish Diseases, 36: 371-388.
4. Pridgeon, J.W., P.H. Klesius, L. Song, D. Zhang, K. Kojima and J.A. Mobley, 2013. Identification, virulence and mass spectrometry of toxic ECP fractions of West Alabama isolates of *Aeromonas hydrophila* obtained from a 2010 disease outbreak. Veterinary microbiology, 164(3): 336-343.
5. Nawaz, M., S.A. Khan, A.A. Khan, K. Sung, Q. Tran, K. Kerdahi and R. Steele, 2010. Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food microbiology, 27(3): 327-331.
6. Boyd, J., J. Williams, B. Curtis, C. Kozera, R. Singh and M. Reith, 2003. Three small, cryptic plasmids from *Aeromonas salmonicida* subsp. *salmonicida* A449. Plasmid, 50(2): 131-44.
7. Majumdar, T., S. Datta, D. Ghosh, S. Dutta, A. Chakraborty, R. Goswami and S. Mazumder, 2007. Role of virulence plasmid of *Aeromonas hydrophila* in the pathogenesis of ulcerative disease syndrome in *Clarias batrachus*. Indian Journal of Biochemistry and Biophysics, 44(5): 401.
8. Bello-Lopez, J.M., E. Fernandez-Rendon and E. Curiel-Quesada, 2010. *In vivo* transfer of plasmidp RAS1 between *Aeromonas salmonicida* and *Aeromonas hydrophila* in artificially infected *Cyprinus carpio* L. Journal of Fish Diseases, 33(3): 251-9.
9. Attéré, S.A., A.T. Vincent, M.V. Trudel, R. Chanut and S.J. Charette, 2015. Diversity and Homogeneity amongSmall Plasmids of *Aeromonas salmonicida* subsp. *Salmonicida* Linked with Geographical Origin. Frontiers in Microbiology, 6: 1274.
10. Marchesi, J.R., T. Sato, A.J. Weightman, T.A. Martin, J.C. Fry, S.J. Hiom and W.G. Wade, 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and Environmental Microbiology, 64(2): 795-799.
11. Zhang, Y.L., C.T. Ong and K.Y. Leung, 2000. Molecular analysis of genetic differences between virulent and avirulent strains of *Aeromonas hydrophila* isolated from diseased fish. Microbiology, 146(4): 999-1009.
12. Kingombe, C.I.B., G. Huys, M. Tonolla, M.J. Albert, J. Swings, R. Peduzzi and T. Jemmi, 1999. PCR detection, characterization and distribution of virulence genes in *Aeromonas* spp. Applied and Environmental Microbiology, 65(12): 5293-5302.
13. Abu-Elala, N., M. Abdelsalam, Sh. Marouf and A. Setta, 2015. Comparative analysis of virulence genes, antibiotic resistance and gyrB-based phylogeny of motile *Aeromonas* species isolates from Nile tilapia and domestic fowl. Letters in Applied Microbiology, 61: 429-436.
14. Oliveira, S.T., G. Veneroni-Gouveia and M.M. Costa, 2012. Molecular characterization of virulence factors in *Aeromonas hydrophila* obtained from fish. Pesquisa Veterinária Brasileira, 32(8): 701-706.
15. Merino, S., A. Aguilar, M.M. Nogueras, M. Regue, S. Swift and J.M. Tomás, 1999. Cloning, sequencing and role in virulence of two phospholipases (A1 and C) from mesophilic *Aeromonas* sp. serogroup O: 34. Infection and Immunity, 67(8): 4008-4013.

16. Gavín, R., S. Merino, M. Altarriba, R. Canals, J.G. Shaw and J.M. Tomás, 2003. Lateral flagella are required for increased celladherence, invasion and biofilm formation by *Aeromonas* spp. FEMS Microbiological Letters, 224: 77-83.
17. Kirov, S.M., B.C. Tassell, A.B. Semmler, L.A. O'Donovan, A.A. Rabaan and J.G. Shaw, 2002. Lateral flagella and swarming motility in *Aeromonas* species. Journal of Bacteriology, 184(2): 547-555.
18. Furmanek-Blaszk, B., 2014. Phenotypic and molecular characteristics of an *Aeromonas hydrophila* strain isolated from the River Nile. Microbiological Research, 169: 547-552.
19. Han, J.E., J.H. Kim, C.H. Choresca, S.P. Shin, J.W. Jun, J.Y. Chai and S.C. Park, 2012. First description of ColE-type plasmid in *Aeromonas* spp. Carrying quinolone resistance (*qnrS2*) gene. Letters in Applied Microbiology, 55: 290-294.