



Identification and Characterization of Virulence-Associated Genes from Pathogenic *Aeromonas Hydrophila* Strains

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ABSTRACT

Aeromonas hydrophila is a freshwater, Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacterium that exists frequently in aquatic environments producing disease, not only to fish but also to human causing gastroenteritis. The present study aims to isolate, identify and characterize *A. hydrophila* isolated from *Oreochromis niloticus* fish in Kafr El-Sheikh governorate, Egypt using selective differential cultural medium (Rimler Shotts agar), morphological and biochemical tests (oxidase, catalase, methyl red, voges proskauer, indole, citrate utilization, gelatin hydrolysis, glucose, triple sugar iron, urease, starch hydrolysis, lactose and trehalose tests). Besides, to search for the presence of the virulence genes in the pathogenic *A. hydrophila* isolates. In the present study we screened the presence of five virulence-associated genes of *A. hydrophila* isolated from diseased cultured fish. The detection of virulence factors of *A. hydrophila* is a key component in determining potential pathogenicity because these factors act multifunctionally and multifactorially. Pathogenesis of *A. hydrophila* was checked by experimental infection to *Oreochromis niloticus* fish together with screening of the five virulence genes which are heat-stable enterotoxin (ast), cytotoxic enterotoxin, hemolysin and aerolysin and heat-labile enterotoxin (alt). The obtained results revealed that the five screened virulence genes were positively correlated with *A. hydrophila* pathogenicity and the presence of virulence genes in pathogenic *A. hydrophila* strains may help in disease diagnosis, prevention and control.

Key words: *Aeromonas hydrophila*, Identification, Characterization, *Oreochromis niloticus*, Virulence genes

INTRODUCTION

Aeromonas hydrophila, belonging to the family *Aeromonadaceae*, is an environmental microorganism which is cosmopolitan in distribution. Mesophilic motile *aeromonads* are ubiquitous and autochthonous aquatic microorganisms occurring in fresh water, sewage and brackish water and in chlorinated and un-chlorinated drinking water. *Aeromonas hydrophila* is a freshwater, Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacterium (Garrity et al., 2006) that occurs frequently in aquatic environments such as surface waters, as well as food. Besides, it is an opportunistic pathogen implicated in human illnesses such as gastroenteritis, wound infections, septicaemia, pneumonia, necrotizing fasciitis and soft tissue conditions, has gained increasing interest (Janda and Abbott, 2010; Oleiwi et al., 2014; Praveen et al., 2016).

Some virulent strains of *A. hydrophila*, under stress conditions can invade most of freshwater fish species infecting them with hemorrhagic septicemia (MAS). In China, MAS has become the most important and dangerous bacterial fish disease to date leading to periodical high economic losses per year (Yang et al., 2016). The pathogenesis of *A. hydrophila* is multifactorial and the virulence factors work together to produce the virulence of this bacterium, these factors include extracellular products and S-layer that help the bacteria to resist the host immunity (Pang et al., 2015).

The pathogenicity of *Aeromonads* has been linked to exotoxins such as cytolytic enterotoxin, hemolysin/aerolysin, lipases and proteases (Yogananth et al., 2009). Hemolysins are exotoxins and the lytic activities on red blood cells are reported to be important for nutrient acquisition and/or for causing anemia (Sarkar et al., 2013). Haemolytic toxins as haemolysin and aerolysin released by *A. hydrophila* and their pathogenicity have been linked to hemagglutinins, adhesins and several hydrolytic enzymes. These toxins enter the tissue in culture and play a significant role in the pathogenesis of the bacteria. ExoPolySaccharides (EPS), importantly, play a very great role for the bacteria and their as they are organic molecules formed by polymerization of organic fractions, carbohydrates, proteins, and humic substances (Sarkar et al., 2013).

The objectives of this study were to (i) isolate, identify and characterize *A. hydrophila* isolated from *Oreochromis niloticus* fish in Kafr El-Sheikh governorate, Egypt, using various culture media, morphological and biochemical tests, and (ii) search for the presence of the virulence genes in the pathogenic *A. hydrophila* isolates. In the present study we screened the presence of five virulence-associated genes of *A. hydrophila* isolated from diseased cultured fish.

MATERIALS AND METHODS

Ethical approval

Animal ethics committee, faculty of veterinary medicine, Kafr El-Sheikh University, Egypt, approved the protocol and conducting of the study.

Isolation and identification of *Aeromonas hydrophila*

A total number of 140 *Oreochromis niloticus* fish were collected from Kafr El-Sheikh governorate farms, Egypt, over the seasons of the year 2015. The collected fish showed petechial haemorrhages externally over the body, fins and particularly in the head region. The peritoneum was swollen with bloody ascitic fluid with gross visible hemorrhages on the internal organs of the affected fish. For bacteriological examination, sterile swabs were collected from the infected parts of the fishes. The collected swabs were firstly pre-enriched on Tryptic Soy Broth (TSB, Oxoid). After which (pre-enrichment on TSB) they were streaked on Rimler Shotts (RS) agar for presumptive identification and incubated at 28 °C for 24 hours. Well-differentiated single bacterial colonies were further streaked onto TSA (TSA, Oxoid) for obtaining pure culture.

Biochemical analysis

For phenotypic identification, pure cultures were subjected to Gram staining and viewed under light microscope (Leica). Further biochemical tests like motility, oxidase, catalase, methyl red, voges proskauer, indole, citrate utilization, gelatin hydrolysis, glucose, triple sugar iron, urease, starch hydrolysis, lactose and trehalose tests, were performed for the identification as well as strain differentiation of bacteria and results were compared with the reference strain of *A. hydrophila* (obtained from the food analysis center, faculty of veterinary medicine, Banha university, Egypt).

Molecular identification by PCR

DNA Extraction using QIA amp kit: DNA Extraction was performed using QIA amp kit (QIAamp: Qiagen inc., USA). Accurately, one ml of the young cell suspension was centrifuged at 10000 g for 10 min at 4°C and the cell pellet was mixed with 600 µl of guanidine hydrochloride buffer (pH 8.0), incubated at room temperature for 30 min and again centrifuged at 10000 x g for 10 min at 4°C. From that, 500 µl of the supernatant was transferred to another tube and mixed with 100% ice cold ethanol and centrifuged at 13000 x g for 20 min at 4°C. The supernatant was discarded and the pellet was washed with 95% and 90% ethanol, respectively followed by centrifugation at 10000 g for 10 min at 4°C. The pellet was then re-suspended in 50 µl of molecular grade water and then stored at -20°C until used as PCR template (Haldar et al., 2005).

Primer sequences used for PCR identification system of *A. hydrophila*: Application of PCR for identification of *A. hydrophila* by 16S rRNA and identification of virulence factors including, heat-stable enterotoxin (ast), cytotoxic enterotoxin, hemolysin and aerolysin (act/hlyA/aer) complex and heat-labile enterotoxin (alt) of the isolated *A. hydrophila* was performed essentially by using Primers (Pharmacia Biotech) as shown in table 1.

Table 1. The primers used for the amplification of different virulence associated genes of *A. hydrophila* isolated from *O. niloticus*

Primers	Target gene	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
A16S1 (F) A16S1 (R)	16S rRNA	5' CTACTTTTGCCGGCGAGCGG '3 5' TGATTCCCGAAGGCACTCCC '3	953	Pinto et al., (2012)
AHS (F) AHS (R)	Ast	5' GACTTCAATCGCTTCCTCAACG '3 5' GCATCGAAGTCACTGGTGAAGC '3	536	Bin Kingombe et al., (2010)
act/hlyA/aer complex (F) act/hlyA/aer complex (R)	act/hlyA/aer complex	5' AGAAGGTGACYACCAAGAACA '3 5' CCACTTCACTTCACCCGGGA '3	400	Balsalobre et al., (2009)
AHL (F) AHL (R)	Alt	5' TGCTGGGCCTGCGTCTGGCGGT '3 5' AGGAACCTCGTTGACGAAGCAGG '3	361	Bin Kingombe et al., (2010)

DNA amplification for the selected virulent genes

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) using 25 µl of PCR mixture. The reaction mix invariably consisted of 10X PCR buffer (100 mM Tris [pH 9.0], 500 mM KCl, 15 mM MgCl₂, and 0.1% gelatin), 10 mM MgCl₂, 100 mM of each dNTP, 25 pmol of each forward and reverse primers and 1.25 U of Taq DNA polymerase. The PCR cycling protocol was applied as following: An initial denaturation at 94°C for three minute, followed by denaturation at 94°C for 60 sec, extension at 72°C for 90 sec for 45 cycles, and final extension at 72°C for three min. Finally, 5 µl of each amplicon was electrophoresed in 1.5% agarose gel (Sigma –USA), stained with ethidium bromide and visualized as well as captured on UV trans-illuminator. A 100 bp DNA ladder was used as a marker for PCR products (Hussain et al., 2013).

Experimental infection

Total Bacterial count: The pour plate method for estimation of *A. hydrophila* strains per one ml, was used in demonstration of the inoculum dose for the experimental studies according to Cruickshank et al. (1975).

Lethal Dose fifty (LD₅₀): A total number of 210 apparently healthy *Oreochromis niloticus* fish weighting 40±0.5 gm, were divided into three main groups in which each group was subdivided into seven subgroups, 10 fish per each group and the seventh group was kept as a control group. All fishes were kept for 15 days under observation prior to injection for accommodation and to confirm that they are free from diseases.

24 hours colony cultures of *A. hydrophila* strains on TSA were used. The colonies were picked up and suspended in sterile saline in a tenfold serial dilution with subsequent incubation at 28 °C for 24 hours for plate counts on TSA. Only the dilutions (10²-10⁷CFU) were used. Each group was intra-peritoneally injected with 0.5 ml/fish of each bacterial dilution. The fishes in the control group were injected with 0.5 ml PBS/fish. All the injected fish were kept for one week post-inoculation for observation. The mortalities were recorded twice/day according to Ibrahim et al. (2011). The freshly dead fishes were moved for further post-mortem examination. The LD₅₀ (the dose which kill 50% of the injected fish) was calculated according to Reed and Muench (1938).

Pathogenicity test: Experimental infection was carried out to determine the pathogenicity of *A. hydrophila* strains using intra-peritoneal route injection according to Li et al. (2011). A total number of 100 apparently healthy *O. niloticus* fish weighting 40±0.5 gm, were divided into five groups, 20 fishes per each. Each fish in the first group was intra-peritoneally injected with 0.2 ml/fish of LD₅₀ dose of *A. hydrophila* strain (A1) which was determined previously (2.3 x 10⁶cfu). Each fish in the second group was intra-peritoneally injected with 0.2 ml/fish of LD₅₀ dose of *A. hydrophila* strain (A2) which was determined previously (2 x 10⁶cfu). Each fish in the third group was intra-peritoneally injected with 0.2 ml/fish of LD₅₀ dose of *A. hydrophila* strain (A3) which was determined previously (1.8 x 10⁶cfu). Each fish in the fourth group (control negative group), was intra-peritoneally injected with 0.2 ml/fish of PBS. Each fish in the fifth group (Control positive group), was intra-peritoneally injected with 0.2 ml/fish of the obtained reference *A. hydrophila* strain. All injected fishes were observed for a period of 28 days post-inoculation. Mortalities were recorded daily and freshly dead fishes were moved for further pm examination and histopathological studies.

Histopathological examination

Specimens from liver, kidney and spleen of experimentally infected fishes were taken. Specimens were fixed immediately in 10% neutral buffered formalin, dehydrated and embedded in paraffin blocks. Paraffin blocks were sectioned at 4-5 µm thickness and stained with Hematoxylin and Eosin (H&E) and examined under light microscope (Leica) using ×200 and ×400 magnification power according to Bancroft and Gamble (2007).

RESULTS AND DISCUSSION

External examination of naturally infected *Oreochromis niloticus* revealed hemorrhages on the external surface and surrounding the anus (Figure 1 and Figure 2) and cutaneous ulcers (Figure 3). The gross lesions displayed in the current study are similar to those reported by several authors (Harikrishnan and Balasundaram, 2005; Sarkar and Rashid, 2012; Yardimci and Aydin, 2011). However, the postmortem findings exhibited the presence of abundant ascetic fluid in the abdominal cavity together with hemorrhagic gas bladder and congested liver (Figure 4). The postmortem findings displayed in the present study are similar to those reported by (Miyazaki and Kaige, 1985; Popovic et al., 2000; Harikrishnan and Balasundaram, 2005). The current postmortem findings may be due to the action of extracellular products of *A. hydrophila* which secretes haemolysin, aerolysin and cytotoxic toxins that possess a haemolytic, cytolytic and enterotoxic activities (Harikrishnan and Balasundaram, 2005).

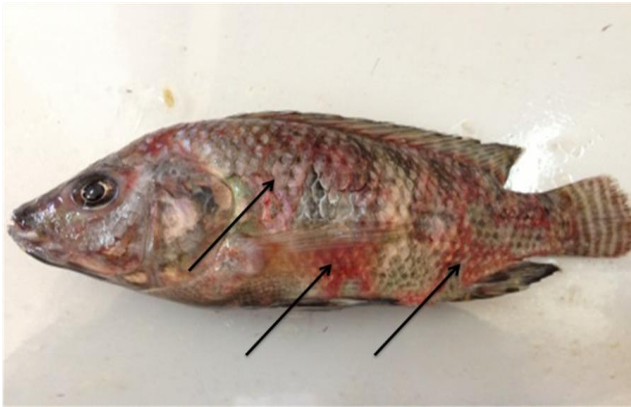


Figure 1. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *A. hydrophila* showing hemorrhagic patches (black arrows) on different parts of the body



Figure 2. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in autumn, naturally infected with *A. hydrophila* showing exophthalmia and blindness (black arrow)

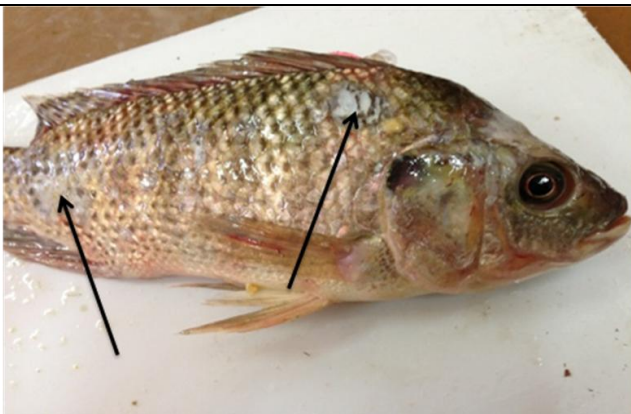


Figure 3. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *A. hydrophila* showing cutaneous ulcers on the body (black arrows)

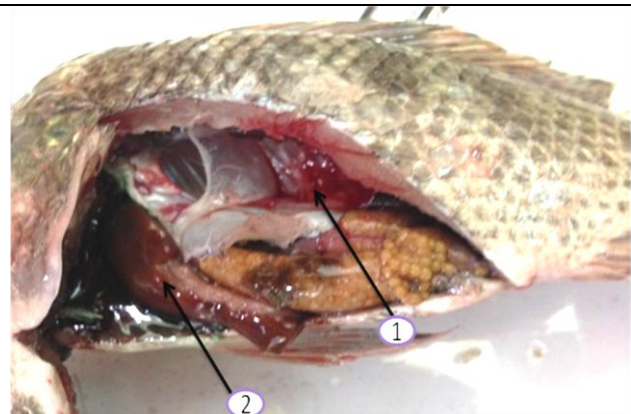


Figure 4. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in autumn, naturally infected with *A. hydrophila* showing hemorrhagic gas bladder (black arrow-1) and congested liver (black arrow-2)

Table 2. Comparison of the phenotypic and biochemical characteristics between the isolated *A. hydrophila* strains and the reference strain

Biochemical Tests	RESULTS			
	<i>Aeromonas hydrophila</i> strains			
	A1	A2	A3	Reference strain
Motility	+	+	+	+
Gram staining	-	-	-	-
Indole	+	+	+	+
MR*	-	-	-	-
VP**	+	+	+	+
Citrate utilization	+	+	+	+
Catalase	+	+	+	+
Triple Sugar Iron	+	+	+	+
Urease	-	-	-	-
Oxidase	+	+	+	+
Lactose	+	+	+	+
Glucose	+	+	+	+
Trehalose	+	+	+	+
Starch Hydrolysis	+	+	+	+
Gelatin Hydrolysis	+	+	+	+

*MR: methyl red test; ** VP: Voges Proskauer

The presumptive identification of the bacteria in the recent study was carried out from the colony morphology over (RS) medium which acts as a selective medium for *A. hydrophila*. They produced deep cream or light yellow colonies with entire margin over the RS-medium. This obtained result is similar to that obtained by Sarkar et al. (2013). The bacteria were observed as Gram-negative motile rods microscopically; the result which coincides with those reported by Cartwright et al. (1994). Biochemical characterizations have proven to be a valuable method for typing and differentiation of bacterial fish pathogens (Sarker et al., 2012). In the present study, based on 15 biochemical tests, a total number of 45 isolates were positively identified as *A. hydrophila*. All strains were positive for catalase, indole test, voges proskauer, oxidase test, citrate utilization, carbohydrate utilization, lactose, trehalose, glucose, starch, gelatine hydrolysis and for triple sugar iron agar medium. The strains are observed to be negative for methyl red and urease test (Table 2). Most of the phenotypic characteristics of the isolates were similar to those reported in Bergey's manual of determinative bacteriology (Holt 1994). Based on the biochemical tests results, all obtained isolates were similar to *A. hydrophila* reference strain. The obtained biochemical results were similar to findings of Jayavignesh et al. (2011). The PCR amplification with *A. hydrophila* specific primers (*A. hydrophila* specific-16S rRNA) identified 45 isolates into *A. hydrophila* species. Three of *A. hydrophila* positive strains were screened for the presence of five virulence genes (*ast*), cytotoxic enterotoxin, (*act/hlyA/aer*) complex and (*alt*) by PCR technique. The results revealed that two of the three screened *A. hydrophila* strains had the five virulence genes (*ast*, *act*, *hlyA*, *aer* and *alt*), while the third strain had only four virulence genes (*ast*, *act*, *hlyA* and *aer*) (Figure 5).

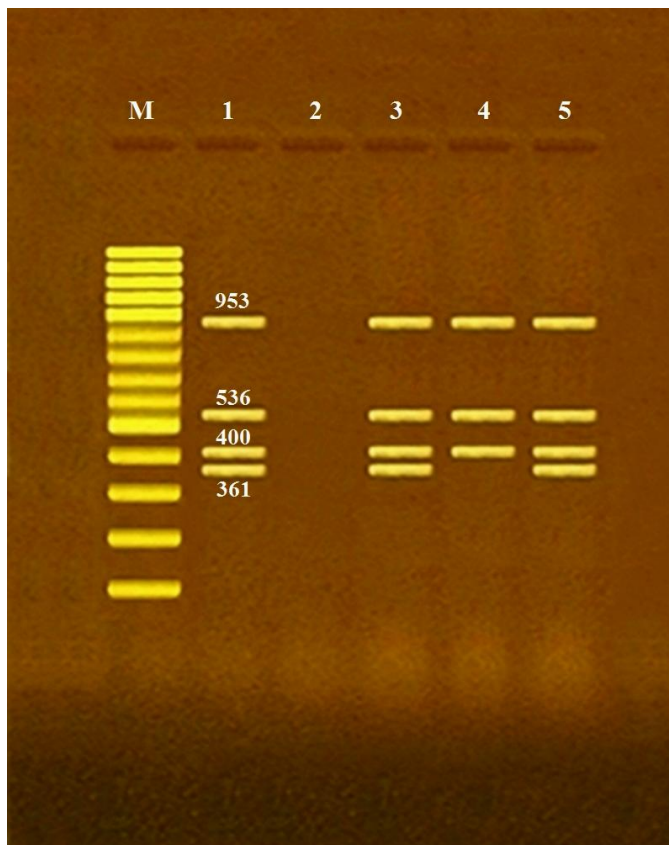


Figure 5. Agarose gel electrophoresis of multiplex PCR of 16S rRNA (953 bp), *ast* (536 bp), *act/hlyA/aer* complex (400 bp) and *alt* (361 bp) virulence genes for characterization of *A. hydrophila*.

Lane M: 100 bp ladder as molecular size DNA marker; Lane 1: Control positive *A. hydrophila* for 16S rRNA, *ast*, *act/hlyA/aer* complex and *alt* genes; Lane 2: Control negative; Lane 3 and Lane 5: Positive *A. hydrophila* strains for 16S rRNA, *ast*, *act/hlyA/aer* complex and *alt* genes; Lanes 4: Positive *A. hydrophila* strains for 16S rRNA, *ast* and *act/hlyA/aer* complex genes

In *Aeromonas* spp., as with all pathogens, disease is the result of complex molecular interactions between bacterium, environment, and host; however, the literature on *A. hydrophila* remains limited by the lack of experimental data on validated members of *A. hydrophila*. While there was numerous virulence factors shared between members of *A. hydrophila* (Rasmussen-Ivey et al., 2016). It has been reported that the five virulence genes (*ast*, *act*, *hlyA*, *aer* and *alt*), obtained in the current study were specific to pathogenic *A. hydrophila* (Cartwright et al., 1994; Paniagua et al., 1990). The virulence of *A. hydrophila* is complex and involves multiple virulence factors, which may work in concert enabling the bacteria to colonize, gain entry, establish, replicate and damage the host tissues and to evade the host defense system and spread, eventually killing the host. Haemolytic toxins; haemolysin and aerolysin released by *A. hydrophila* may be used as a marker of pathogenicity of *A. hydrophila* (Al-Maleky et al., 2011).

The LD₅₀ experiments in the present study revealed that the concentration 10⁶cfu was the most potent dilution causing 50% mortalities within (24-48 hr). This result is similar to Pachanawan et al. (2008) and Omar (2014) but, disagrees with Viji et al. (2011) and Li et al. (2011) who obtained LD₅₀ at concentration of 10³ -10⁵cfu. The differences in LD₅₀ between different authors may be due to the number of the virulent genes in *A. hydrophila* strains, pathogen properties as well as size and weight of fish species. The LD₅₀ experiments with *A. hydrophila* strains in *O. niloticus* revealed a higher mortality rate for A1, A2 and A3 strains which may be due to the higher number of virulence genes, five virulence genes in A1 and A3 and 4 virulence genes in A2 which may be responsible for the production of extracellular toxins as well as, the synergistic effects conferred by combination of several virulence genes (Viji et al., 2011).

The clinical signs of experimentally infected *O. niloticus* with *A. hydrophila* strains revealed that the fish became lethargic and anorexic, with increased respiratory frequency and mucus production. Abnormal movement and loss of

balance were observed. The injected fish were characterized by swollen abdomen, red mouth, hemorrhages on the external surface and surrounding the anus. Hemorrhages on the external body surface may be attributed to the hemolytic effect of the exotoxins, haemolysin (α -hemolysins) and aerolysin (β -hemolysins). Both hemolysins induce pore-formation in the cell membrane of the RBCs, leading to haemolysis Zhang et al. (2000); Singh et al. (2008); Singh et al. (2010) and Hidalgo and Figueras (2013).

The Pathogenesis of motile aeromonas septicemia is complex and associated with multiple virulence factors. The recorded mortalities of the experimentally infected fishes may be attributed to the presence of more than one virulent gene in the same strain as well as the synergistic effects conferred by combination of several virulence genes including (ast) and (alt), which increase the vascular permeability of the gut causing detachment of the intestinal mucosa and cytotoxic enterotoxin which possess a hemolytic, cytolytic, and enterotoxic activities Harikrishnan and Balasundaram (2005).

The histopathological findings of the experimentally infected *O. niloticus* in the present study were represented by diffuse degenerative lesions especially in parenchymatous organs such as liver and kidneys. The liver showed diffuse hepatocytes vacuolation, degeneration of the pancreatic portion and multifocal necrotic foci (Figure 6). Similarly, the kidney showed diffuse degenerative lesions extended to coagulative necrosis of the renal tubules (Figure 7). These findings were nearly similar to those reported by Harikrishnan and Balasundaram, (2005); Oliveira et al. (2012). The findings of histopathological changes might be attributed to different virulence genes (hemolysin, aerolysin, cytotoxic enterotoxin and cytotoxic heat stable enterotoxin). These virulence factors exhibit hemolytic, cytotoxic and enterotoxic activities that play a role in the establishment of *A. hydrophila* infection (Li et al., 2011).

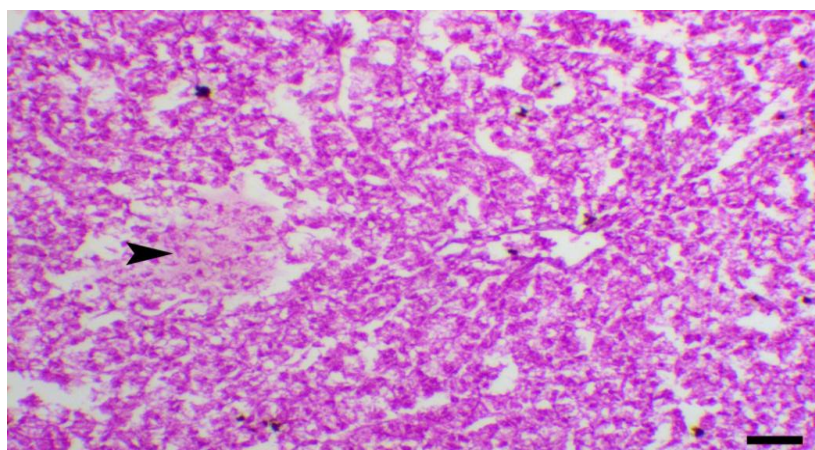


Figure 6. Liver of *Oreochromis niloticus* infected with *A. hydrophila* showing necrotic foci (arrowhead), H&E, bar=100 μ m, \times 200

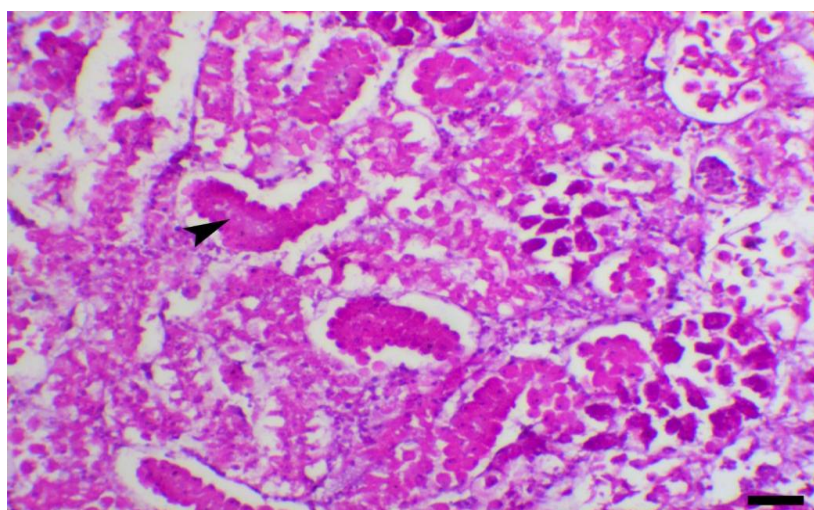


Figure 7. Kidney of *Oreochromis niloticus* infected with *A. hydrophila* showing coagulative necrosis of the renal tubules (arrowhead), H&E, bar=100 μ m, \times 200

CONCLUSION

A. hydrophila is a Gram-negative, rod-shaped, facultatively anaerobic bacterium; a pathogen, not only to fish but also to human causing gastroenteritis. *A. hydrophila* strains possess a wide range of virulence factors enabling the pathogen to evade the host defense mechanisms, spread and eventually kill the host. Virulence genes act as a key component in determining the potential pathogenicity of the micro-organism, acting multifunctionally and multifactorially and can be used for virulence typing of *A. hydrophila* isolates. In the present work, the five screened

virulence genes were positively correlated with *A. hydrophila* pathogenicity and the presence of virulence genes in pathogenic *A. hydrophila* strains may help in diagnosis, prevention and control of the disease.

Competing interests

Authors have declared that there is no competing interest.

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