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Fish diseases

Molecular Studies on *Aeromonas Species* Isolated from Freshwater Fishes Collected from Delta Region, Egypt

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ABSTRACT

The current study was carried out to identify suspected causes of septicemia in naturally infected freshwater fishes collected from different fish farms at different localities in Menofiya and Kafr El-Sheikh Provinces, Egypt. Fishes (n=120) were examined clinically and upon post-mortem. Bacteriological isolation and identification of the bacterial pathogens using traditional and molecular methods were demonstrated. Based on the phenotypic and biochemical characterization using API20E, API 20 NE and VITEK® 2 compact, the isolated bacteria were identified as Aeromonads (A. hydrophila and A. veronii). Molecular characterization was accomplished through polymerase chain reaction (PCR) and confirmed by sequencing and phylogenetic analysis. The high molecular identity and close phylogenetic relationship confirmed that the isolates were genetically homologous to A. hydrophila and A. veronii. Thus, this study proves that motile aeromonads remain important bacterial pathogens from aquaculture point of view in Egypt, which requires regular and permanent examination of cultured fishes to resist mass mortalities which lead to economic losses.

Keywords: Freshwater Fishes, A. hydrophila, A. veronii, Sequencing, phylogenetic analysis.

INTRODUCTION

Nowadays, yield of fish by aquaculture is nearly equivalent to capture fisheries yield, so aquaculture will become the predominant source of seafood through the coming years (FAO, 2018). Increased production leads to the possibility of increasing the incidence of diseases and consequently occurrence of economic havoc (Zhou *et al.*, 2020). Motile Aeromonads are commonly inhabitant in miscellaneous aquatic environments, thus fish are continually infected with bacterial diseases. Aeromonads are part of the normal microflora found in fish intestine. When the fish is subjected to stress factors due to inappropriate environmental conditions and human interference during harvesting, sorting and transport of fish, it results in bacterial infection. (Austin and Austin, 2016). Aeromonas species are mainly infecting freshwater fishes and sometimes marine fishes. Also, there are aquatic animals that are susceptible to infection by these bacteria for instance lobsters, shrimp, oysters, frogs, and eels (Yano *et al.*, 2015). Aeromonas veronii has been considered one of the serious threats facing Oreochromis niloticus (Dong et al. 2017). Aeromonas hydrophila is considered a prevailing pathogen in the Egyptian aquaculture, and is responsible for mass mortalities in the per-acute phase. Ulcers of skin and fins with abdominal dropsy and eye lesions were the most revealed signs in the acute phase that causes considerable economic casualties in aquaculture industry because of decreased probability of fish survival after treatment (Elsheshtawy *et al.*, 2019; Abdel-Latif and Khafaga, 2020). Thus, the aim of this

study was directed to determine the most common Aeromonas species recovered from naturally infected fishes collected from different regions in Menofiya and Kafr El-Sheikh Provinces, Egypt. Also, morphological and biochemical identification of the bacterial strains and the molecular characterization by polymerase chain reaction (PCR). Finally, DNA sequencing, and phylogenetic relationship were executed to confirm the identity of Aeromonas isolates retrieved from fishes.

MATERIALS AND METHODS

Fish sample:

A total number of 120 cultured fishes (60 ***Oreochromis niloticus***, 40 ***Mugil cephalus*** and 20 ***Clarias gariepinus***) were collected from different fish farms at different regions in (Menofiya and Kafr El-Sheikh Provinces) (Table1). Fishes were transported alive to the Department of Fish Diseases at AHRI (Animal Health Research Institute), Dokki, Giza and exposed to clinical and PM examination.

Gross clinical examination:-

Naturally infected fishes have been clinically examined to investigate any clinical abnormalities acc. to Noga, (2010) and Austin and Austin, (2016).

Post mortem examination:-

Necropsy was performed for detection of PM lesions acc. to Noga, (2010) and Austin and Austin, (2016).

Isolation and morpho- biochemical Identification of Aeromonas Spp.:-

Samples were taken from fish's organs (gills, liver, kidney, and spleen) under full aseptic conditions. The inoculums were streaked onto tryptic soya agar plates (TSA; Difco, Detroit, MI) and incubation was performed at 37 °C for 24 hrs and one separate colony was selected from each plate and sub-cultured on Rimler-Shotts (RS) agar medium (RS; Difco, Detroit, MI) (Shotts and Rimler, 1973) and Aeromonas base agar medium (Merck, Germany). Incubation of plates was performed at 37 °C for 24 hrs. Full identification scheme preliminarily based on colonial characteristics, Gram stain and biochemical characters of the strains according to Austin and Austin, (2016). Isolates were kept at −80 °C in BHIB including 10% glycerol for further molecular identification. The biochemical characters of the identified bacterial isolates were further confirmed using API 20 E and API 20 NE Kit according to (Buller, 2004) and VITEK® 2 compact (BioMérieux).

Molecular Identification, DNA sequencing, and phylogenetic relationships:

The two different isolates were submitted to identification by16SrRNA. The isolates were inoculated into brain heart infusion broth (BHIB) and incubated for 24 hrs. DNA was extracted using PathoGene-spin™ DNA Extraction Kit acc. to the manufacturer's instructions. PCR was accomplished using oligonucleotide primer for general gram negative 16srRNA gene. The PCR protocol was as follows an initial denaturation at 95°C / 5 min, followed by 35 cycles 94°C / 2 min; 35 cycles of 94°C /30 s, 55°C / 30 s, and 72°C /30 s; 72°C / 5 min. QIAquick PCR Product extraction kit has been used to extract PCR products (Qiagen, Valencia). Sequencing was done using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) and then it was purified using Centrisep spin column. DNA sequencing was performed by Applied Biosystems3130 genetic analyze r(HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al.,* 1990), initially was conducted to confirm sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of BioEdit and Phylogenetic tree was created using neighbor-joining method in MEGA 7 (Tamura *et al.,* 2013).

Results:

Gross clinical and PM examination of Fishes:

Clinical examination of different fish species including (***Oreochromis niloticus, Mugil cephalus*** and ***Clarias gariepinus***) revealed darkness of skin, hemorrhages in different parts of the fish's body including the base of fins, tail, and rot of fins, abdominal distension, ulcers and sloughing of fish scales. While, the post-mortem examination of examined fishes revealed septicemia represented by gills congestion and viscera, enlargement of different internal organs and some cases exhibited that liver paleness and intestine .

Bacterial examinations:-

Morphological, cultural characters, and Biochemical identification:

Colonies isolated from fish's organs streaked onto Tryptic soya agar, Rimler-Shotts (RS) agar medium and Aeromonas base agar media. Colonies of Aeromonas spp. on Tryptic soya agar appeared round, convex, shiny and

creamy. On Rimler-Shotts (RS) agar medium produced yellow colonies after 24 hrs of incubation at 37 °C, while on Aeromonas base agar media produced small, dark green colonies with a dark center. All isolated Aeromonas spp. were Oxidase and Catalase positive and they gave negative results toward urea hydrolysis and H2S production. Most Aeromonas spp. showed gram-negative coccobacilli to rod-shaped when examined by Gram stain and motile. The data existed in (Table 2) illustrated full identification scheme of Aeromonas Spp. using API 20E system.

Results of VITEK® 2 compact system

Two strains were identified using Vitek®2 compact system. Two isolates were identified as A. hydrophila and A. veronii, with a probability of 99%.

Molecular identification, DNA Sequencing and Phylogenetic relationship:

QIAquick PCR Product extraction kit has been used to extract PCR products. DNA sequencing was performed by Applied Biosystems3130 genetic analyzer, a BLAST® analysis (Basic Local Alignment Search Tool), initially was conducted to confirm sequence identity to GenBank accessions. From the sequence of 16SrRNA gene, the two isolates sequence were Aeromonas hydrophila and Aeromonas veronii, and were submitted to Genbank data base under accession no. MW494427and MW564031, respectively (Figure 1, 2 and 3).

Table 1: Showing Species of fish, Region and No. :-

Species of fish	Region (Province)	No.
Oreochroms niloticus	Menofiya and Kafr El- Sheikh	60
Mugil cephalus	Menofiya and Kafr El- Sheikh	40
Clarias gariepinus	Menofiya and Kafr El- Sheikh	20
Total		120

Table (2): illustrated the full identification scheme of Aeromonas Spp. using API 20E system.

	Aeromonas hydrophilla	**Aeromonas veronii**
Gram stain	-ve	-ve
Motility	Motile	Motile
Growth on Rimler-Shotts agar medium	yellow colonies	yellow colonies
API 20E reactions		
(ONPG) ß-galactosidase	+ve	+ve
(ADH) Arginine dihydrolase	+ve	+ve
(LDC) Lysine decarboxylase	+ve	+ve
(ODC) Ornithine decarboxylase	-ve	+ve
(CIT) Citrate utilization	+ve	+ve
(H2S) H2S production	-ve	-ve
(URE) Urea hydrolysis	-ve	-ve
(TDA) Tryptophan deamination	-ve	+ve
(IND) Indol production	+ve	+ve
(VP) Acetoin production	+ve	+ve
(GEL) Gelatin hydrolysis	+ve	+ve
(GLU) Glucose fermentation	+ve	+ve
(MAN) Mannitol	+ve	+ve
(INO) Inositol	-ve	-ve
(SOR) Sorbitol	-ve	-ve
(RHA) Rhamnose	-ve	-ve
(SAC) Sucrose	+ve	+ve
(MEL) Melibiose	+ve	-ve
(AMY) Amygdalin	-ve	+ve
(ARA) Arabinose	+ve	-ve
(Oxidase) Cytochrome oxidase	+ve	+ve

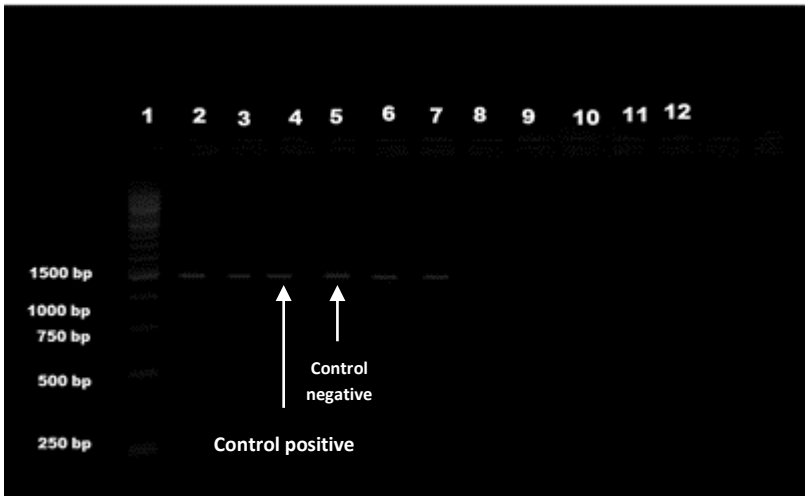

Figure (1): Agrose gel electrophoresis of 16SrRNA gene
Lane (1):1000 bp DNA Ladder.
Lanes (2-3): positive isolates for 16srRNA gene.
Lanes (4): Control positive *Escherichia coli* (ATCC; 25922).
Lanes (5): Control negative Staphylococcus aureus subsp. Aureus *(*ATCC; 6538).

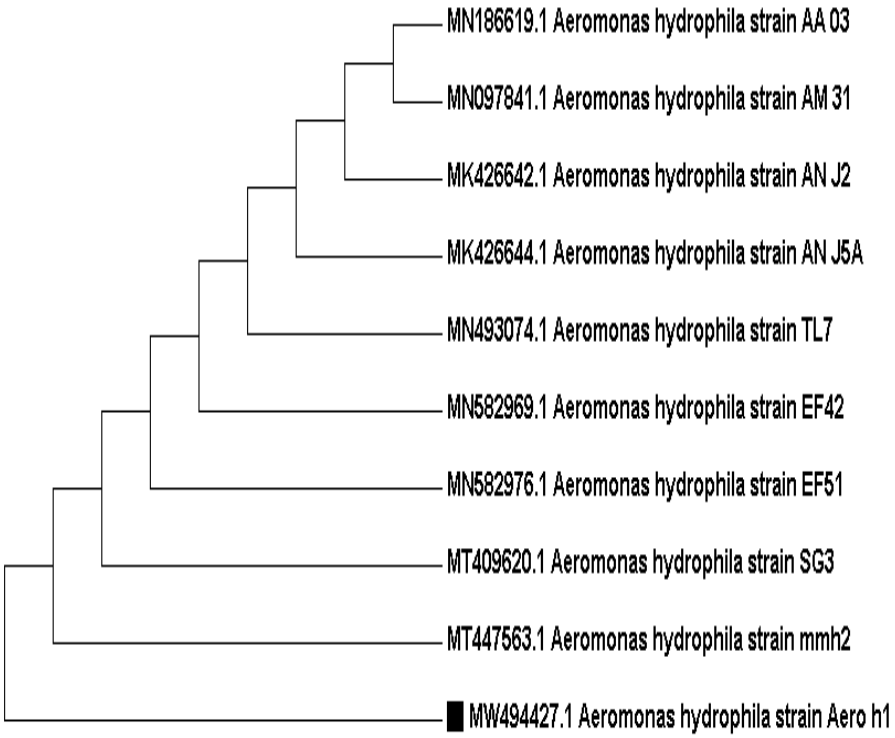

Figure (2): Phylogenetic tree of Aeromonas hydrophila strain by general gram negative 16SrRNA gene demonstrating the relationships of Aeromonas hydrophila with related species. The neighbor-joining method has been used to create phylogenetic tree.

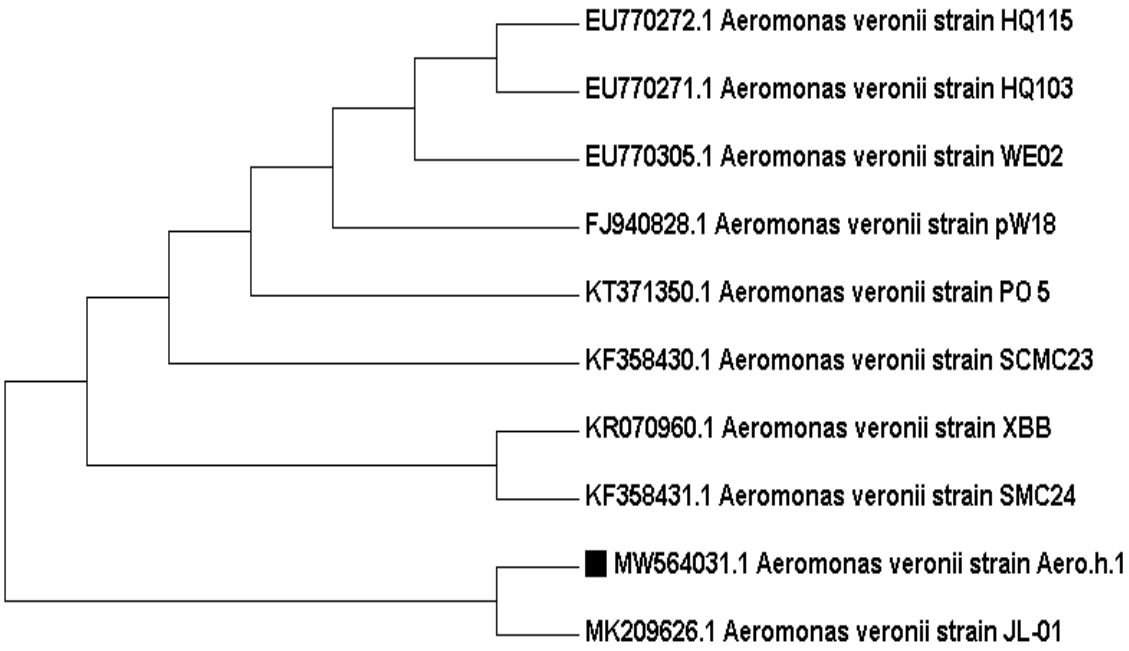

Figure (3): Phylogenetic tree of Aeromonas veronii strain by general gram negative 16SrRNA gene demonstrating the relationships of Aeromonas veronii with related species. The neighbor-joining method has been used to create phylogenetic tree.

DISCUSSION

Disease outbreaks caused by Aeromonas species are considered a common episode and lead to significant mortality and huge economic losses in cultured Nile tilapia globally (Mzula *et al.,* 2019; Raj *et al.,* 2019). Motile Aeromonads are Gram-negative, non-spore forming rods belonging to Aeromonadaceae family within the class Gammaproteo bacteria. These facultative anaerobic bacteria are very common in Aquatic environments and have wide range of susceptible host (Abdelhamed *et al.,* 2019). Regardless of the common contributions of different Aeromonas species in causing diseases in aquatic animals, A. hydrophila is the leading cause of disease outbreaks in fresh water fishes resulting in food insecurity and economic casualties globally (Aboyadak *et al.,* 2015; Baumgartner *et al.,* 2017). In the current study, a total of 120 freshwater fishes were collected from different regions in (Menofiya and Kafr El-Sheikh Provinces). Fishes were subjected to clinical, PM and bacterial identification for the presence of Aeromonas species. In addition to the morpho-biochemical identification of the bacterial strains, the molecular characterization by polymerase chain reaction (PCR) was done. Also, sequencing, and phylogenetic relationships were executed to confirm the identity of Aeromonas species isolated from fishes. Clinical examination of different fish species revealed darkness of skin, hemorrhages in different parts of the fish's body including the base of fins, tail, and rot of fins, abdominal distension, ulcers and sloughing of fish scales. Our results are in agreementt with those previously reported (Janda and Abbott, 2010; Parvez and Mudarris, 2014; AlYahya *et al.,* 2018; Abd El Latif *et al.,* 2019; Claudiano *et al.,* 2019; Algammal *et al.,* 2020). The fishes were further examined for postmortem lesions of the internal organs. The collected data showed that in some cases, congestion of gills and visceral organs, enlargement of liver, spleen, and kidney and in some cases paleness of liver and intestine were recorded. These results are in agreement with that formerly obtained in many studies (Parvez and Mudarris, 2014; El-Barbary and Hal, 2016; Elsayed *et al.,* 2018; Elsheshtawy *et al.,* 2019). Hemorrhagic septicemia was also reported in other studies in which relationship between stress factors and incidence of disease was revealed. This relationship indicated that opportunistic pathogens, such as Aeromonas specis had the ability of causing infection only when the fish were exposed to stress factors resulting from environmental conditions and had poor appetite for feed, the unconsumed feed allowing that virulent Aeromonas hydrophila (vAh) with ample nutrients to spread immediately and quickly, which would lead to incidence of MAS outbreak (Eissa et al. 2015; Zhang *et al.,* 2020).

Although, Motile Aeromonas Septicemia in fish can be presumed by clinical lesions, however, appropriate confirmation of the infection needs to isolation and identification of the causative agent. Isolation and identification of Aeromonas strains based on the colonial characters on selective media and the results of the biochemical reactions. The characteristic colonies of Aeromonas species appeared round, convex, shiny, and creamy on TSA media, while appeared as yellow colonies on RS-media and on Aeromonas base agar media produced small, dark green colonies with a dark center. The isolated Aeromonas spp were positive for Oxidase and Catalase, while gave negative results for urea hydrolysis and H2S production. Most of the isolates appeared as gram-negative coccobacilli to rod-shaped on Gram-stain. These outcomes are in accordance with that described previously (Beaz-Hidalgo and Figueras, 2013; Elhady and Ahmed, 2014; Hassan *et al.,* 2017; Monir *et al.,* 2017; Matter *et al.,* 2018; Salem et al. 2020). API 20 E, API 20 NE and VITEK 2 compact system were used to confirm the results of biochemical reactions. However, with the progress of molecular biology, arrays of genomic techniques have been developed for accurate and fast related species identification in aquaculture. DNA-sequence-based identification mainly based on 16S rRNA and housekeeping genes (Chatterjee and Haldar, 2012). This isolate Aeromonas hydrophila (MW494427) had high nucleotide identity with other isolates MN567598.1 isolated from Epinephelus lanceolatus, KY003115, KY003116 and KY003121 which were isolated from Lates calcarifer, LC369692.1, LC369698.1, LC369706. This isolate Aeromonas veronii (MW564031) had similar nucleotide identity with strain MK209626.1 Which recovered from Monopterus albus, and high nucleotide identity with KR070960.1 which was isolated from Carassius auratus ,KF358430.1, KF358431.1 that were isolated from Grass carp Each, EU770272.1, EU770271 that were isolated from carp and FJ940828, EU770305 that were isolated from fish pond also, EU770305 which was recovered from seafood effluent.

CONCLUSION

Aeromonads are pathogenic bacteria which give rise to huge economic losses in fish farms and thus affects the aquaculture industry in Egypt. Therefore, rapid, and trustworthy diagnosis of Aeromonads considers an important aspect of the disease management programs.

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