



Prevalence and virulence characteristics of *Aeromonas* species isolated from fish farms in Egypt

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Abstract

Background/Objective: Fish is one of the most valuable food for human consumption. However, fish may also act as a source of foodborne pathogens including *Aeromonas* species which caused a serious threat to a public health concern. This study aimed to investigate the prevalence and virulence characteristics of *Aeromonas* species isolated from fish farms in Egypt.

Methods: A total of 100 random samples of freshwater fish represented by Nile tilapia and Mugil cephalus (50 of each) were collected from different fish farms in Kafr El-Sheikh governorate and examined bacteriologically and biochemically for the presence of *Aeromonas* species. Multiplex PCR was done to detect some virulence-associated genes in *Aeromonas hydrophila* isolates.

Results: The obtained results revealed that the incidence of *Aeromonas* species in Nile tilapia and Mugil cephalus were 32 (64%) and 25 (50%), respectively. The most prevalent *Aeromonas* species isolated from Nile tilapia were *Aeromonas caviae* 13 (40.6%), *Aeromonas hydrophila* 8 (25%), *Aeromonas sobria* 7 (21.9%), *Aeromonas veronii* 3 (9.4%), and *Aeromonas fluviialis* 1 (3.1%), while from fresh Mugil cephalus were *Aeromonas sobria* 11 (44%), *Aeromonas caviae* 7 (28%), *Aeromonas hydrophila* 5 (20%), and *Aeromonas veronii* 2 (8%). The isolated *Aeromonas hydrophila* concealed some virulence genes responsible for their pathogenicities such as aerolysin gene (*aerA*) and cytotoxic enterotoxin gene (*altA*).

Conclusion: The hygienic measures should be applied to prevent fish contamination with *Aeromonas* species.

Keywords: *Aeromonas* species, Nile tilapia, Mugil cephalus, Virulence genes, Prevalence

1. Introduction

Fish is considered as the major source of valuable food for human consumption due to it is easily digested, high palatability, a rich source of nutrients and provides a good balance of high-quality protein, vitamins, and minerals (Pal et al, 2018). In contrast, fish may also act as a source of foodborne pathogens including *Aeromonas* species that have been known as emerging foodborne pathogens of serious threat to a public health concern (Igbinsosa et al, 2012). *Aeromonas* species are associated with food poisoning and some human diseases as gastrointestinal infections and extra-intestinal infections such as skin and soft-tissue infections, traumatic wound infections, and lower respiratory tract/urinary tract infections (Batra et al, 2016).

The genus *Aeromonas* belongs to the Aeromonadaceae family and includes a group of Gram-negative bacteria which characterized by oxidase-positive, facultatively anaerobic, glucose-fermenting, mainly motile rods and form many exoenzymes (Pund and Theegarten 2008). *Aeromonas* species includes 32 and 12 species and subspecies, respectively among them; *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas veronii*, and *Aeromonas sobria* (Janda and Abbott 2010). The pathogenicity of *Aeromonas* species is attributed to the release of various virulence factors that are associated with exotoxins, cytotoxic and hemolytic activities which causes adhesion and colonization of

mucosa, followed by fluid accumulation or epithelial change are likely events leading to human disease (Daskalov, 2006).

The present study aimed to determine the incidence of *Aeromonas* species in fresh Nile tilapia and Mugil cephalus isolated from the different fish farms in Kafr El-Sheikh governorate and to detect the most virulence factors which responsible for their pathogenicity.

2. Materials and methods

This study was conducted after under the ethical approval from the Experimental Animals Care Committee in compliance with guidelines of the University of Kafrelsheikh.

2.1. Collection of fish samples

A total of 100 samples of freshwater fish represented by Nile tilapia and Mugil cephalus (50 of each) with the average weight of 150-250 g were randomly collected from different fish farms in Kafr El-Sheikh governorate during winter 2017 from January to April. The collected samples were packaged in a sterile plastic bag, sealed, and then directly transferred immediately in a cooled isolated box under aseptic conditions to the laboratory for bacterial isolation and identification.

2.2. Sensory assessment of fish samples

The sensory assessment of fresh fish was accessed as recommended by (Davis, 1995) according to the following criteria: general appearance which included the examination of the eye, cornea, and gills (5, extremely desirable; 1, extremely unacceptable); odor (10, extremely desirable; 1, extremely unacceptable); and color (10, no discoloration; 1, extreme discoloration).

2.3. Preparation of fish samples

Each fish sample was laid on its side over a sterile plate by sterile forceps, the dorsal, pectoral, and ventral fins were removed by a sterile scissor and forceps. Scales were removed by sterile scalpel and the body surface was sterilized using a hot spatula. The sterilized surface was removed by sterile scissors and forceps under aseptic condition and then 5 g of the back muscles were transferred aseptically into a sterile homogenizer tube containing 45 ml of sterile 1% peptone water. The contents were homogenized for 2.5 minutes at 14000 rpm, then allowed to stand for 5 minutes according to APHA (1992).

2.4. Bacterial isolation

From the prepared homogenate, 1 ml was transferred into a sterile test tube containing 9 ml of brain heart infusion broth (BHI) as an enrichment broth then incubated at 28°C for 24 hours. After incubation, a loopful from the enrichment broth was streaked onto Aeromonas Agar medium and incubated aerobically at 37°C for 18-24 hours according to Austin (1999). Suspected colonies (Translucent, pale green colonies 0.5-3.0 mm diameter) should be confirmed as presumptive Aeromonas species by performing the further identification.

2.5. Morphological and biochemical identification

Pure cultures of the isolates were morphologically identified including Gram staining, and motility of the isolated Aeromonas and biochemically identified using the following tests; esculin hydrolysis, oxidase, arginine hydrolysis, indole, methyl red, Voges Proskauer, citrate utilization, urease, hydrogen sulfide production, nitrate reduction, gelatin liquefaction, Ornithine decarboxylase, oxidation-fermentation, L- lysine decarboxylase, arginine decarboxylase, β -galactosidase and sugars fermentation according to (Koneman et al., 1994; Macfaddin, 2000). Suspected Aeromonas colonies were biochemically confirmed according to Table 1.

2.6. Detection of virulence genes of Aeromonas hydrophila by multiplex PCR

The extraction of the bacterial DNA was carried out using QIA amp kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and as previously described (Mansour et al., 2019). PCR amplification of *Aeromonas hydrophila* virulence genes [16S rRNA, aerolysin (*aerA*) and cytotoxic enterotoxin (*altA*)] was done using the following primers: 16SrRNA sense 5' ATG GCTGGG TAATAATGGG '3 and antisense 5' GCCTGTAGAGCTCGATC '3; *aerA* sense 5' TTAGCTCGAGAGGAAGCCCA '3 and antisense 5' TAGGGATAGGAGATGTCAG '3; and *altA* sense 5' TCAACA CCATCACCGACGT '3 and antisense 5' ATCGAACTTGAACAG GGCA '3 according to (Aslani and Hamzeh, 2004; Venkataiah et al., 2013). Multiplex PCR was carried out in 50 μ l reaction containing 0.4 μ mol of 16S rRNA-F and 16S rRNA-R, 0.4 μ mol of *aerA*-F and *aerA*-R, 0.4 μ mol of *altA*-F and *altA*-R, 0.2 mmol of each dNTPs, 1.2 unit of Taq polymerase, mM MgCl₂ in 1X PCR buffer with 10 ng of template DNA. Amplification included initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 2 min and extension at 72°C for 2 min and a final extension at 72°C for 10 min. Amplified DNA fragments were analyzed by 2% of agarose gel electrophoresis in 1x TBE buffer stained with ethidium bromide and captured as well as visualized on

a UV transilluminator. A 100 bp plus DNA Ladder was used to determine the fragment sizes according to Allam et al., 2019.

3. Results and discussion

3.1. Sensory assessment of fish samples

Depending on the grading scheme, all the examined samples were fresh and fit for human consumption (Table 2). This sensory evaluation should be associated with the bacterial examination to give accurate judgment. No significant difference in general appearance or odor was noticed among Nile tilapia and *Mugil cephalus* samples, but there was a highly significant difference in the flavor ($p < 0.05$). There was a preference for the flavor of Nile tilapia over *Mugil cephalus* and this may be attributed to the higher fat percentage in *Mugil cephalus* which may slightly affect the taste of consumers (Aman et al. 2017).

3.2. Prevalence of Aeromonas species in fresh fish samples

The obtained data in Table 3 revealed that out of 50 Nile tilapia and 50 *Mugil cephalus* samples analyzed, 32 (64%) Nile tilapia and 25 (50%) *Mugil cephalus* were contaminated with Aeromonas species based on the colonial character on Aeromonas Agar media and biochemical identification. The higher incidence of Aeromonas species in Nile tilapia than *Mugil cephalus* could be attributed to the difference in the type of water, fish species, bad handling, and manipulations during catching, storage, and transportation for Nile tilapia. The obtained results agreed with El-ghareeb et al. (2019) who revealed that 43 isolates of Aeromonas strains were isolated from 75 Nile tilapia fish samples with a percentage of 57.33%. While 38 isolated of Aeromonas strains were isolated from 75 *Mugil cephalus* samples with a percentage of 50.67%. However, the results of these study were less than reported by Ebeed et al. (2017) who examined 100 samples of Nile tilapia and *Mugil cephalus* (50 for each) were collected from different fish markets in Kafr El-sheikh governorate and found that the Aeromonas species percentage was 68% and 62% in Nile tilapia and *Mugil cephalus* samples, respectively. The variations of Aeromonas species incidence could be attributed to various species, sampling time and place, geographical range, and post-capture contamination and this agrees with Hafez et al., (2018).

The frequency distribution of isolated Aeromonas species of examined Nile tilapia samples was *Aeromonas caviae* 13 (40.6%), *Aeromonas hydrophila* 8 (25%), *Aeromonas sobria* 7 (21.9%), *Aeromonas veronii* 3 (9.4%) and *Aeromonas fluviialis* 1 (3.1%). While the frequency distribution of isolated Aeromonas species of examined *Mugil cephalus* samples was *Aeromonas sobria* 11 (44%), *Aeromonas caviae* 7 (28%), *Aeromonas hydrophila* 5 (20%), and *Aeromonas veronii* 2 (8%) (Table 3). It can be concluded from these results that *Aeromonas caviae*, *Aeromonas hydrophila*, *Aeromonas sobria* were the most predominant identified pathogenic Aeromonas species isolated from Nile tilapia and *Mugil cephalus* samples. Whereas, the higher incidence of Aeromonas species in Nile tilapia and *Mugil cephalus* samples were *Aeromonas caviae* and *Aeromonas sobria*, respectively. In agreement, Igbinsa et al. (2012) showed that *Aeromonas caviae*, *Aeromonas hydrophila*, and *Aeromonas sobria* were the main cause of Aeromonas associated with human disease.

3.3. Detection of virulence factors of Aeromonas hydrophila

Aeromonas hydrophila is one of the most pathogenic Aeromonas species associated with several foodborne outbreaks caused by the ingestion a raw fermented fish in Egypt and all over the world. *Aeromonas hydrophila* isolation can be confirmed by isolation of 16S rRNA (Venkataiah et al. 2013). *Aeromonas hydrophila* can release a variety of virulence factors that are associated with enterotoxic, cytotoxic, and hemolytic activities which cause adhesion and colonization of mucosa, followed by fluid accumulation or epithelial change are likely events leading to human disease (Daskalov, 2006; Janda and Abbott, 2010).

Table (A): Biochemical tests for identification of *Aeromonas* species according to Macfaddin, (2000).

Test	<i>Aeromonas hydrophila</i>	<i>Aeromonas sobria</i>	<i>Aeromonas Caviae</i>	<i>Aeromonas veronii</i>	<i>Aeromonas fluvialis</i>
Motility	+	+	+	+	+
Esculine hydrolysis	+	-	-	-	-
Oxidase	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+
Indole	+	+	+/-	+	-
Methyle red	+/-	-	+/-	+/-	-
Voges Proskauer	+/-	+/-	-	-	+/-
Citrate utilization	+/-	+	+/-	+	+/-
Urease	-	-	-	-	+/-
Hydrogen sulphide production	+	+	-	+/-	+
Nitrate reduction	+	+/-	-	+/-	-
Gelatin liquefaction	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	+/-
L- lysine decarboxylase	+	+	-	+	+/-
arginine decarboxylase	+	+/-	+/-	+	-
β - galactosidase	+	+	+	+	+
Glucose fermentation	+	+	+	+	+
Sucrose fermentation	+	+	+	+	-
Rhamnose fermentation	+	-	-	-	-
Mannose fermentation	+	+	+/-	+/-	+
Arabinose fermentation	+/-	-	+	+/-	+
Inositol fermentation	-	-	+/-	-	-
Sorbitol fermentation	+/-	-	-	-	-
Oxidative/Fermentative	Fermentative	Fermentative	Fermentative	Fermentative	Fermentative

* Positive: (+)

* Negative: (-)

* Variable: (+/-)

Table 2. Means values of sensory assessment of fish samples.

Fish species	Parameters	Minimum	Maximum	Mean \pm SE
Nile tilapia	General appearance (5 points)	3	5	4.3 \pm 0.07 ^c
	Odor (10 points)	7	10	9.14 \pm 0.85 ^a
	Flavor (10 points)	7	10	9.22 \pm 0.11 ^a
Mugil cephalus	General appearance (5 points)	4	5	4.28 \pm 0.06 ^c
	Odor (10 points)	8	10	9.60 \pm 0.08 ^a
	Flavor (10 points)	7	10	8.74 \pm 0.07 ^b

Mean values within the same column with different superscript letters are significantly different at (P<0.05).

Table 3. Prevalence of *Aeromonas* species in the examined fresh fish samples.

Fish samples (n=50)	Positive samples*	<i>Aeromonas hydrophila</i> **	<i>Aeromonas caviae</i> **	<i>Aeromonas fluvialis</i> **	<i>Aeromonas sobria</i> **	<i>Aeromonas veronii</i> **
Nile Tilapia	32 (64%)	8 (25%)	13 (40.6%)	1 (3.1%)	7 (21.9%)	3 (9.4%)
Mugil cephalus	25 (50%)	5 (20%)	7 (28%)	-	11 (44%)	2 (8%)

*Percentages were calculated according to the number of examined samples.

**Percentages were calculated according to the number of positive samples.

The incidence of *Aeromonas hydrophila* strains depend on multiplex PCR based on the amplification of gene 16S rRNA was 8 (16%) and 5 (10%) in Nile tilapia and *Mugil cephalus* samples, respectively. The results obtained using multiplex PCR revealed that 16S rRNA was present in all the isolates with a percentage of 100% and these results indicated that 16S rRNA can be used as a specific target for the detection of *Aeromonas hydrophila*.

The occurrence of virulence genes of *Aeromonas hydrophila* strains isolated from examined fish samples were 10 *Aeromonas hydrophila* strains of the tested 13 isolates carried aerolysin gene

(*aerA*) at the expected product size 841 bp (6 isolates from Nile tilapia with a percentage of 64.2% and 4 isolates from *Mugil cephalus* with a percentage of 30.8%). A total of 9 *Aeromonas hydrophila* strains of the tested 13 isolates carried cytotoxic enterotoxin gene (*altA*) at the expected product size 425 bp (5 isolates from Nile tilapia with a percentage of 38.5% and 4 isolates from *Mugil cephalus* with a percentage of 30.8%). Therefore, *Aeromonas hydrophila* strains contain one or more from the virulence genes which are responsible for its pathogenicity.

Conclusion

The presence of *Aeromonas* species in Nile tilapia and *Mugil cephalus* does not only affect fish quality, reduce shelf life and acceptability but it is also considered a public health hazard to the consumers. *Aeromonas hydrophila* harbored virulence factors responsible for their pathogenicity. Therefore, hygienic measures should be applied to control the microbial contamination either in the aquatic environment or during fish transportation until reach to consumers.

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