Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 64 (2): 34-42. Jan 2020
DOI: 10.5455/ajvs.70246

Molecular Characterization of Some Virulence Genes and Antibiotic Susceptibility of *Aeromonas Hydrophila* Isolated from Fish and Water

Rasha A. Sonkol, Helmy A. Torky, Samy A. Khalil
Department of Microbiology, Faculty of Veterinary Medicine, Alexandria University, Egypt

Key words:
Fish, water, antibiotic susceptibility, PCR, Virulence gene

***Correspondence to:**
rashasonkol@gmail.com

Article History
Received: 02 Oct 2019
Accepted: 15 Dec 2019

ABSTRACT

The present study was done to verify the presence of certain virulence genes in A. hydrophila isolated from fish and water samples collected from different farms in three governorates (Kafr El-sheikh, El-Behera and Damietta), in addition to study the antibiotic susceptibility of isolated A. hydrophila strain. The obtained results showed that the overall prevalence of A. hydrophila i examined fish samples was 25% with high incidence in Kafr El-sheik farms (30%) followed by El-Behera (25%) and Damietta farms (20%). Antibiotic sensitivity of *A. hydrophila* strains showed the highest antimicrobial resistance to ampicillin, amoxicillin and Cephradine and highly susceptible to Florfenicol and Doxycycline. Polymerase chain reaction (PCR) assessment was done to detect the presence of three virulence genes in the ten biochemically identified *A. hydrophila* and found that the most frequent virulence gene detected in *A. hydrophila* strains isolated from both fish and water samples was aerolysin gene by incidence 100% at molecular size 326 bp followed by haemolysin gene 30% at molecular size 1500bp, and all isolates completely free from shigatoxin 2. PCR not only detect bacterial pathogens in fish but also differentiate between different bacterial strains and serotypes. The detection of virulence genes in *A. hydrophila* is critical in determining pathogenicity of the organism and subsequently the possible drug targets for prevention of its infection.

1. INTRODUCTION

Aeromonas hydrophila is gram-negative bacteria, ubiquitous in aquatic environment and has been implicated in the aetiologies of a variety of systemic and localized diseases in fish (Austin and Austin, 2012). Most important microbial pathogens affecting cultured and feral fishes worldwide and resulted in million dollars loses to aquaculture (Sreedharan et al. 2012). The symptoms of Aeromoniasis include; skin hemorrhages, red sores, body ulceration, off-appetite, lethargy, popeyes, dropsy and fins rot (Hu et al. 2012).

Motile Aeromonads have been isolated and characterized from aquatic environment (Rathore et al. 2005). In addition, *A. hydrophila* were recovered from fish and water samples (Rathore et al., 2006). The United States Environmental Protection Agency has incorporated Aeromonas species in the contaminant candidate list of emerging water borne pathogens (Borchardt et al. 2003).

Multiple antibiotic resistance (MAR) from A. hydrophila is a worldwide problem caused by the misuse of antibiotics (Odeyemi et al., 2012 and Sharma et al., 2015). The antibiotic resistance A. hydrophila may be transmitted from fish to humans by infection with pathogenic bacteria and causes health problems in human beings (Didugu et al., 2016).

The presence of resistance genes in mobile elements, such as plasmids, transposons and integron, facilitates their rapid spread among bacteria; these mobile elements, could be transmitted among bacteria via three pathways, including lateral DNA transfer, namely transformation, transduction and conjugation (Romero et al. 2012). The microbial resistance in aeromonads is chromosomally mediated; however, β-lactamases may be coded by plasmids or integrons (Aravena-Romɑn et al., 2012).

Virulence of Aeromonads is considered to be multifactorial including cytotonic heat-labile (alt),

and cytotonic heat-stable enterotoxins (ast), hemolysin (hly), aerolysin (aer), and shigatoxin 2 (Puthucheary et al. 2012). Aerolysin and hemolysin are the most studied genes in Aeromonas spp. (Yousr et al., 2007). These virulence factors contribute to its pathogenicity and provide the ability to attach to host cells in the development of diseases. These factors can be used for detection and characterization of the bacteria (Singh et al., 2008).

Aerolysin is a hemolytic toxin protein secreted by *Aeromonas hydrophila*. The active aerolysin protein binds to the specific glycophospha-tidylinositol (GPI)-anchored proteins on the target surface of eukaryotic red blood cells and forms pores in the cell membrane causing lysis (Parker et al. 1994). Aerolysin is significant and a stable molecular marker to detect the possible virulent *A. hydrophila*.

Conventional identification of *A. hydrophila* is achieved through standard biochemical tests that are time consuming, laborious, not always conclusive and failed to accurately differentiate between different strains and serotypes (Citarasu et al., 2011). In contrast, molecular based identification can accurately identify different bacterial strains and serotypes quickly, more specifically and in a more sensitive and reliable way. Polymerase chain reaction (PCR) was successfully applied to not only detect bacterial pathogens in fish but also differentiate between different bacterial strains and serotypes (Balsalobre et al. 2009; Hu et al. 2012; Oliveira et al. 2012).

Therefore, this study was designed to study the prevalence of *A. hydrophila* virulent strains with detection of certain virulence genes (*areo, hyl* and Stx_2) in fish and water collected from different farms in three governorates (El-Behera, Kafr-Elsheikh and Damietta). In addition, study the antibiotic sensitivity pattern of isolated *A. hydrophila* strains.

2. MATERIAL AND METHODS

2.1. Samples collection:

A total of 200 of apparently and diseased fishes including (common carp, Oreochromis niloticus, Mugil cephalus, Sliver carp and Oreochromis mossambicus) were collected in tank with water from three provinces represented as (100 from El-Behera farms, 50 from Kafr El-sheikh farms and 50 from Damietta farms). The fish samples were kept in tanks partially filled with the same water of the pond then transported to microbiological lab, Faculty of Veterinary Medicine, Alexandria University. The samples were taken according to (Bullar, 2004). Swaps from water and internal organ (liver, kidney, gills and spleen) of each fish were inoculated on Tryptic soya broth then incubated at 28^{o}C for 24 hours according to (Austin and Austin, 2012).

2.2. Isolation and identification of *Aeromonas hydrophila*

A loop full from each broth tube was streaked onto the following media; MacConkey's agar, Rimler and Schotts (R-S agar) (Oxoid) then incubated at 28 ^{o}C for 24 hours. Purified isolates were used as stocks for further morphological and biochemical identifications according to (Noor El Deen et al. 2014).

2.2.1. Morphological and biochemical characterization according to (Cruickshank et al., 1979).

Bacterial films were prepared from each suspected purified isolate and stained with Gram's stain then examined under the bright field microscope with the oil immersion lens.

2.2.2. Phenotypic identification according to (Yogananth et al., 2009).

Aeromonas hydrophila were identified biochemically to species level by using many tests such as; Kovac's oxidase, oxidation and fermentation, catalase, indole, methyl red test, urease test, haemolysin production, triple sugar iron agar, voges proskauer test, reduction of nitrate to nitrite, H_2S production, and cultures which matched typical reaction of *Aeromonas hydrophila* were confirmed as *Aeromonas hydrophila*.

2.3. Antibiogram of *Aeromonas hydrophila* according to (NCCLS, 2008).

The antimicrobial susceptibilities of Aeromonas hydrophila were performed for ten biochemically identified isolates on Mueller Hinton agar plates (Oxoid) using disc diffusion method. Ten antibiotic disc namely Oxytetracycline OT (30 µg), Florfenicol FF (10 µg), Ciprofloxacin CIP (5 µg), Norfloxacin NOR (10µg), Amoxicillin AX (25 µg), Ampicillin AM (10 µg), Cephradine CE (30 µg), Erythromycin E (15 µg), Sulphamethoxazole-Trimethoprim STX (25 µg) and Doxycycline DO (30 µg) were utilized. After period of 24 hour incubation the zone of inhibition were compared and measured according to manufacturer's instruction (NCCLS, 2008). The result was interpreted as sensitive, intermediate and resistant according to the reference values in the following table

Antibiotic and symbol	Disc content	Resistant	Intermediate	Sensitive
Oxytetracycline (OT)	30 µg	≤ 14	15 - 18	≥ 19

Florfenicol (FF)	10 µg	≤ 14	15 – 18	≥ 19
Ciprofloxacin (CIP)	5 µg	≤ 15	16 - 20	≥ 21
Norfloxacin (NOR)	10 µg	≤ 15	16 - 20	≥ 21
Amoxicillin (AX)	25 µg	≤ 13	14 - 17	≥ 18
Ampicillin (AM)	10 µg	≤ 13	14 - 17	≥ 18
Cephradine (CE)	30 µg	≤ 14	15 - 17	≥ 18
Erythromycin (E)	15 µg	≤ 13	14 - 17	≥ 18
Sulfamethoxazole 23.75 + trimethoprim 1.25 (STX)	25 µg	≤ 10	11 - 15	≥ 16
Doxycycline (DO)	30 µg	≤ 12	13 - 15	≥ 16

2.4. Polymerase chain detection of virulence genes:

PCR was used to detect the presence of Aerolysin (*aero*), haemolysin (*hyl*) and shigatoxin 2 *(STx2*) virulence gene in ten biochemically identified isolates of *A. hydrophila* isolated from fish and water samples.

2.4.1. Oligonucleotide primers used in cPCR:

They have specific sequence and amplify a specific product as shown in following Table.

2.4.1.1. Extraction of DNA: According to QIA amp DNA mini kit instructions

2.4.1.2. Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) code No. RR310A kit

Oligonucleotide primers used in cPCR: They have specific sequence and amplify a specific product as shown in following table.

2.4.1.3. Cycling conditions of the primers during PCR Temperature and time conditions of the three primers during PCR according to specific authors and Emerald Amp GT PCR master mix (Takara) kit.

2.4.1.4. Agarose gel electrophoreses according (Sambrook et al., 1989):

Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask , it was heated in a microwave to dissolve all granules with agitation, and allowed to cool at 70 ^{o}C , then 0.5 mg /ml ethidium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization. The comb was then removed and the electrophoresis tank was filled with TBE buffer. 20 ml of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

	Virulence genes	Oligonucleotide Sequence (5-3)	Sequence Length (bp)	References
1	**Haemolysin (*hly*)**	CTATGAAAAAACTAAAAATAACTG CAGTATAAGTGGGGAAATGGAAAG	**1500 bp**	**Yousr et al., 2007**
2	**Aerolysin (*aero*)**	CACAGCCAATATGTCGGTGAAG GTCACCTTCTCGCTCAGGC	**326 bp**	**Singh et al., 2008**
4	***Stx2***	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	**779 bp**	**Dipineto et al., 2006**

4. RESULTS AND DISCUSSION

In developing coast countries such as Egypt, aquaculture is very important as a good source for animal protein suitable for human consumption (Abbas et al., 2017). Uncontrollable bacterial diseases threatened this source especially those caused by drug resistance bacteria and highly virulent bacteria such as *A. hydrophila* (Mendez et al., 2012). *A. hydrophila* is one of the major sources of disease complications for farmed fishes.

Data illustrated in Table (1) showed that the overall prevalence of *A. hydrophila* from examined fish and water were 25 and 20%, respectively. The highest incidence of *A. hydrophila* in fish and water reported in Kafr El-sheikh farm by incidence 30 and 24 %, respectively followed by El-Behera farm (25 and 20%) and Damietta farm (20 and 16%).

Figure (1): yellow colonies of *A. hydrophila* on R-S media and some biochemical identification photo

Table (1): Incidence of Aeromonas hydrophila isolated from fish and water samples from different three provinces.

Farm	Aeromonas hydrophila			
	Fish		Water	
	No.	%	No.	%
El-Behera	25	25	20	20
Kafr El-sheikh	15	30	12	24
Damietta	10	20	8	16
Total	**50**	**25**	**40**	**20**

Table (2): Result of antibiotic susceptibility of different antibiotics in vitro against ten *A. hydrophila* strains.

AB disc / No.	OT 30 µg	FF 10 µg	CIP 5 µg	Nor 10 µg	AX 25 µg	AM 10 µg	CE 30 µg	E 15 µg	STX 25 µg	DO 30 µg
1	15 (I)	17 (I)	13 (R)	14 (R)	0 (R)	0 (R)	13 (R)	8(R)	7 (R)	7 (R)
2	23 (S)	20 (S)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	15(I)
3	20 (S)	21 (S)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	10 (R)	14 (I)
4	24 (S)	22 (S)	12 (R)	13 (R)	0 (R)	0 (R)	0 (R)	0 (R)	27 (S)	14 (I)
5	22 (S)	28 (S)	25 (S)	22 (S)	0 (R)	0 (R)	0 (R)	18 (S)	27 (S)	15 (I)
6	25 (S)	24 (S)	20 (S)	21 (S)	8 (R)	7 (R)	9 (R)	14 (I)	20 (S)	22 (S)
7	25 (S)	20 (S)	26 (S)	23 (S)	0 (R)	0 (R)	0 (R)	8 (R)	20 (S)	23 (S)
8	21 (S)	22 (S)	16 (I)	16 (I)	9 (R)	10 (R)	13 (R)	7 (R)	9 (R)	14 (I)
9	15 (I)	17 (I)	13 (R)	14 (R)	0 (R)	0 (R)	14 (R)	8 (R)	7 (R)	17 (S)
10	17 (I)	20 (S)	14 (R)	16 (I)	0 (R)	0 (R)	0 (R)	0 (R)	12 (I)	15 (I)

R= Resistant **S= Sensitive** **I= intermediate**

Table (3): Distribution of virulence genes in the ten *Aeromonas hydrophila* isolates from fish and water, (isolates 1-7) from fish, (isolates 8-10) from water.

Isolate No	*Aero*	*hyl*	*Stx2*
1	+	-	-
2	+	+	-
3	+	+	-
4	+	-	-
5	+	-	-
6	+	-	-
7	+	-	-
8	+	+	-
9	+	-	-
10	+	-	-
Total	**10**	**3**	**0**
%	**100**	**30**	**0**

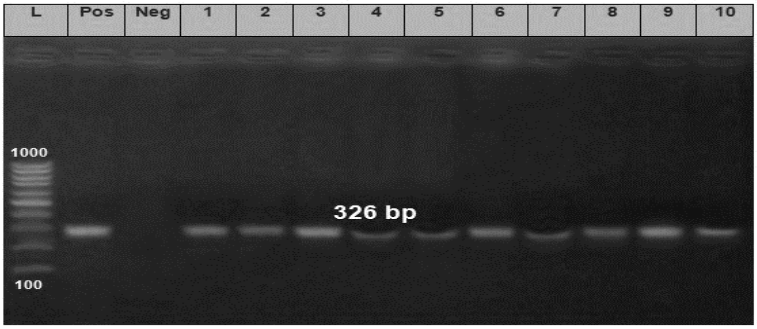

Fig (2): 1.5 % Gel electrophoresis of PCR product showing Aerolysin gene of *A. hydrophila* at molecular size 326 bp for ten isolates. L: Molecular Weight Marker (100- 1000bp). Pos: Positive control (at 326 bp). Neg: Negative control. Lane (1-10): positive for *aero* gene of *A. hydrophila*.

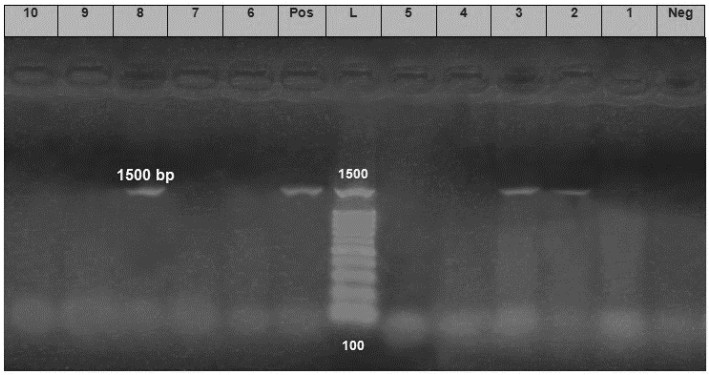

Fig (3): 1.5 %Gel electrophoresis of PCR product showing haemolysin gene of *A. hydrophila* molecular size 1500 bp for ten isolates. L: Molecular Weight Marker (100- 1500 bp). Pos: Positive control (at 1500 bp). Neg: Negative control. Lane (2, 3, 8): positive for *hyl* gene of *A. hydrophila*.

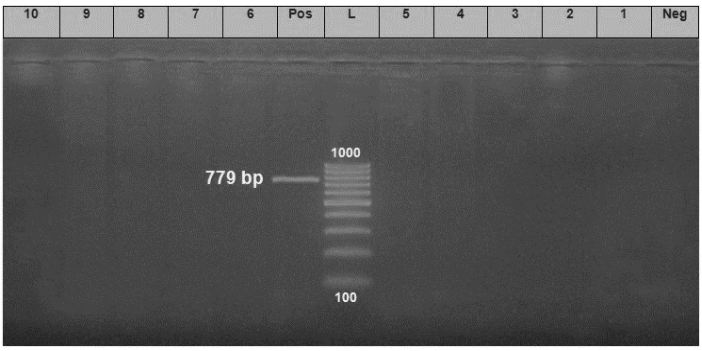

Fig (4): 1.5% Gel electrophoresis of PCR product showing for *Stx2* gene of *A. hydrophila* at molecular size 779 bp for ten isolates. L: Molecular Weight Marker (100- 1000 bp). Pos: Positive control (at 779 bp). Neg: Negative control. All lane negative for *Stx2* virulence gene.

Nearly similar results obtained by (Ramadan et al. 2018 and Mansour et al. 2019a) they reported that incidence of *A. hydrophila* in Mugil cephalus and Oreochromis niloticus collected from Dakahliya and kafr El-sheik were 37 and 35%, respectively. In addition, (Noor El Deen et al. 2014 and El-Bahar et al., 2019) were reported that the prevalence of *A. hydrophila* in examined 40 and 80 Oreochromis niloticus fish collected from different farms in Kafr El-sheik, Egypt were 25 and 27.5 %, respectively.

The highest incidence of *A. hydrophila* using PCR technique was reported by Aboyadak et al. (2015) where confirm that *A. hydrophila* was the main cause of disease outbreak in tilapia farms in Egypt with an incidence of 75% in Kafr-El sheikh Governorate using PCR technique.

The antimicrobial resistance frequencies of the isolated *A. hydrophila* (n = 10) against 10 antimicrobials are presented in Table 2. *Aeromonas hydrophila* isolates exhibited higher resistance to AX, AM and CE (100%) followed by E (80%), CIP (60%), STX (50%) and Nor (50%). None of the isolates exhibited antimicrobial resistance against OT and FF.

Our results of antibiogram for A. hydrophila similar to (El-Bahar et al., 2019) who reported that *A. hydrophila* isolates were highly susceptible to Doxycycline and Florfenicol and all the isolates were resistant to Ampicillin and Amoxicillin. While, *A.*

hydrophila strain isolated from Mugil cephalus in Dakahliya governorate were highly susceptible to Ciprofloxacin and highly resistant to cefoxitin (100%) followed by Ampicillin (84%) (Ramadan et al. 2018).

The test strain of *A. hydrophila* strains were highly resistant to Ampicillin, Amoxicillin and Cephradine may indicate production of beta lactamase, which is consistent with the findings of (Daood 2012; Revina et al. 2017) who found that all *A. hydrophila* isolates recovered from diseased carp and sea trout were 100% resistant to Amoxicillin, Ampicillin, and cephalexin. More than 90% of the tested isolates were highly susceptible to Florfenicol and Doxycycline and thus, these antibiotics are the first choice for the treatment of such infections.

Virulence genes act as a key component in determining the potential pathogenicity of the microorganism, acting multifunctionally and multifactorially and can be used for virulence typing of *A. hydrophila* isolates (Citarasu et al., 2011; Oliveira et al., 2012). PCR method used for diagnostic purposes of cytotoxin-encoding genes of aerolysin (*aer*) and haemolysin (*hly*) Ullmann et al. (2005).

Aerolysin is the major contributor to the virulence of pathogenic Aeromonas strains (Iacovache et al. 2016). Aerolysin is a pore-forming toxin that binds to receptors on the target cell membrane. After proteolytic activation, this toxin induces pore or channel formation, leading to the destruction of membrane permeability, osmotic lysis, and cell death. While, Hemolysin (hlyA) is known to induce hemolysis by destroying RBC cell membranes inducing anemia (Tomas 2012).

Concerning the profile and distribution of the selected virulence genes from the ten *A. hydrophila* isolates is presented in Table (3). The aerolysin (*aer*) gene was detected in 100% (10/10) of the isolates isolated from fish and water, with the expected amplicon observed at 326 bp (Fig 2). The hemolysin (*hyl*) gene was present in 30 % (3/10) of the isolates with a specific band observed at 1500 bp (Fig 3), distributed as follow (two positive isolated from fish and one positive isolated from water. While, we could not detect shigatoxin 2 (*STX2*) gene in all studied A. hydrophila strains (Fig 4).

Our findings revealed that the most frequent virulence gene detected in ten identified A. hydrophila was aerolysin (*aer*) 100% followed by *haemolysin* 30%. These results agree with (Mansour et al. 2019b) who reported that the incidence of aerolysin (*aer*) and haemolysin (*hyl*) were 86 and 24 %, respectively from 21 strains out of 35 strains of *A. hydrophila* identified. In addition, (Ramadan et al. 2018) reported that the *aerA* gene was the most frequently identified virulence gene (70%) of *A. hydrophila* isolated from Mugil cephalus from Dakahliya governorate, whereas no isolates possessed *Stx*2. Singh et al., (2008) reported that aerolysin A virulence gene found in all isolated *A. hydrophila* strains from fish demonstrated hemorrhagic septicemia. Hence, there is a strong positive correlation between the pathogenicity of *A. hydrophila* and the presence of aerA gene. In contrary, Ruhil et al., (2015) reported that most common virulence genes found in all isolates of *A. hydrophila* isolated from fresh water fish in Malaysia was *hly* (95%).

Finally, from the previous results, it can be concluded that Kafr El-sheikh was the highest incidence for *A. hydrophila* followed by El-Behera and Damietta governorate. Aerolysin virulence gene was the most frequent virulence gene detected in A. hydrophila isolated from fish and water samples followed by haemolysin gene and they both contributes to *A. hydrophila* pathogenicity. Florfenicol and Doxycycline were the drugs of choice for the Aeromonas hydrophila infection, while Amoxicillin and Ampicillin are contraindicated due to high resistance.

REFERENCES

Abbas, E., Soliman, T., El-Magd, M. 2017. Phylogeny and DNA Barcoding of the Family Sparidae Inferred from Mitochondrial DNA of the Egyptian Waters. Journal of Fisheries and Aquatic Science, 12:73–81.

Aboyadak, I.M., Nadia, G.M.A., Goda, A.A., Aboelgalagel, W.H., Alnokrashy, A. 2015. Molecular detection of Aeromonas hydrophila as the main cause of outbreak in tilapia farms in Egypt. J. Aquac. Mar. Biol., 2(6): 00045. DOI: 10.15406/jamb.2015.02.00045.

Aravena-Romɑn, M., Inglis, T.J.J., Henderson, B., Riley, T.V., Changa, B.J. 2012. Antimicrobial susceptibilities of Aeromonas strains isolated from clinical and environmental sources to 26 antimicrobial agents. Antimicrob. Agents Chemother., 56:1110-2.

Austin, B. Austin D.A. 2012. Bacterial fish pathogens. Disease of farmed and wild fish, third edition, pp: 112-115.

Balsalobre, L.C., Dropa, M., Matte, G.R., Matte, M.H. 2009. Molecular detection of enterotoxins in environmental strains of Aeromonas hydrophila and Aeromonas jandaei. Journal of Water and Health, 7(4): 685-91.

Borchardt, M.A., Stemper, M.E., Standridge, J.H. 2003. Aeromonas isolates from human diarrheic stool and groundwater compared by pulsed-field gel electrophoresis. Emerg. Infect. Dis. 9(2): 224-228.

Bullar, N.B. 2004. Bacteria from Fish and Other Aquatic Animals: A Practical Identification Manual. CBI Publishing, Cambridge, pp: 83-85.

Citarasu, T., Alfred, D.K., Velmurugan, S. 2011. Isolation of Aeromonas hydrophila from Infected Ornamental fish Hatchery during Massive Disease Outbreak. International Journal of Current Research, 2 (1): 037-41.

Cruickshank, R., J.P. Duguid, B.P. Marmian and R.H.A. Swain, 1979. Medical Microbiol. 2: 121-123. The practice of medical Microbiol 12 ed., Churchill th Livingstone, Edinburgh, London.

Daood, N. 2012. Isolation and antibiotic susceptibility of Aeromonas spp. from freshwater fish farm and farmed carp (Dam of 16 Tishreen, Lattakia). Damas. Univ. J. Basic Sci., 28(1):27–39.

Didugu, H., Krishnaiah, N., Rao, M.T. 2016. Ramanipushpa RN. Antibiogram of Aeromonas Species Isolated From Livestock Products. Int. J. Sci. Environ. Technol., 5 (2): 475–8.

Dipineto, L., Santaniello, A., Fontanella, M., Lagos, K., Fioretti, A., Menna, L.F. 2006. Presence of Shiga toxin-producing Escherichia coli O157:H7 in living layer hens. Letters in Applied Microbiology 43:293–295.

El-Bahar, H.M., Nadia, G.A., Ibrahim, M.A., Samy, A.K., Madiha, S.I. 2019. Virulence genes contributing to Aeromonas hydrophila pathogenicity in Oreochromis niloticus. International Microbiology, https://doi.org/10.1007/s10123-019-00075-3.

Hu, M., Wang, N., Pan, Z.H., Lu C.P., Liu, Y.J. 2012. Identity and Virulence Properties of Aeromonas Isolates from Diseased Fish, Healthy Controls and Water Environment in China. Letters in Applied Microbiology, 55: 224-233.

Iacovache, I., Carlo, S.D., Cirauqui, N., Peraro, M.D., Goot, V.D., Zuber, B. 2016. Cryo-EM structure of aerolysin variants reveals a novel protein fold and the pore-formation process. Nat Commun 7:12062. https://doi.org/10.1038/ncomms12062

Mansour A., Mahfouz, N. B., Husien, M.M., El-Magd, M. A. 2019a. Molecular identification of aeromonas hydrophila strains recovered from kafr Elsheikh fish farms. Slov. Vet. Res. 56 (Suppl 22): 201–208.

Mansour A., Nadia B.M., Mona M.H., Amira, A. E., Eman M. M. 2019b. Molecular characterization and pathogenicity evaluation of Aeromonas hydrophila strains isolated from cultured tilapia Oreochromis niloticus in Egypt. Indian J. Fish., 66(2): 93-100.

Mendez, J., Reimundo, P., Pérez-Pascual, D. 2012. An Overview of Virulence-Associated Factors of Gram-Negative Fish Pathogenic Bacteria, Health and Environment in Aquaculture, Dr. Edmir Carvalho (Ed.), 133-56.

NCCLS (National Committee for Clinical Laboratory Standard) 2008. Performance standards for antimicrobial disc susceptibility tests: approved standard, National Committee for Clinical Laboratory Standard of Antimicrobial Susceptibility, Document M2- AS, Pennsylvania, and USA.

Noor El Deen, A.E., Sohad, M.D., Azza H.M.H., Hakim, A.S.2014. Studies on Aeromonas hydrophila in Cultured Oreochromis niloticus at Kafr El Sheikh Governorate, Egypt with Reference to Histopathological Alterations in Some Vital Organs. World Journal of Fish and Marine Sciences 6 (3): 233-240.

Odeyemi, O., Asmat, A., Usup G. 2012. Antibiotic's resistance and putative virulence factors of Aeromonas hydrophila isolated from estuary. J. Microbiol. Biotechnol. Food Sci., 1 (6): 1339.

Oliveira, S., Veneroni-Gouveia G., Costa, M. 2012. Molecular Characterization of Virulence Factors in Aeromonas hydrophila Obtained from Fish. Pesquisa Veteriinaria Brasileira, 32 (8): 701–6.

Parker, M.W., Buckley, J.T., Postma, J.P.M., Tucker, A.D., Leonard, K., Pattus, F., Tsernoglou, D. 1994. Structure of the Aeromonas toxin proaerolysin in its water-soluble and membrane-channel states. Nature 367:292–295.

Puthucheary, S.D., Puah, S.M., Chua, K.H. 2012. Molecular Characterization of Clinical Isolates of Aeromonas species from Malaysia. Plos One, 7(2): 1-7.

Ramadan H., Ibrahim, N., Samir, M., Abd El-Moaty, A., Gad, T. 2018. Aeromonas hydrophila from marketed mullet (Mugil cephalus) in Egypt: PCR characterization of b-lactam resistance and virulence genes. Journal of Applied Microbiology, 1629-1637.

Rathore, G., Singh, V., Kumar, G., Swaminathan, T.R., Mahanta, P.C. 2006. Antibiotic sensitivity and characterization of Aeromonas hydrophila isolated from fish and water samples. J. Ecophysiol. Occup. Hlth. 6:41-43.

Rathore, G., Swaminathan, T.R., Abidi, R., Mahanta, P.C., Kapoor, D. 2005. Isolation and characterization of motile Aeromonads from aquatic environment. Indian J. Fish, 52: 241-248.

Revina, O., Avsejenko, J., Cirule, D., Valdovska, A. 2017. Antimicrobial resistance of aeromonas spp. isolated from the sea trout (Salmo trutta l.) in Latvia. Res Rural Dev 1:271–275.

Romero, J., Feijoo, C.G., Navarrete, P. 2012. Antibiotics in aquaculture use, abuse and alternatives, health and environment in aquaculture. In: Carvalho ED, Silva RJ, David GS, editors. Health and environment in aquaculture. InTech., p.159-98.

Ruhil H.H., Hassan, M.D., Ong, B.L., Abdelhadi, Y.M., Nur Hidayahanum, H., Sharifah, R.M., Nora Faten, A.M., Kuttichantran, S., Milud Alsaid. 2015. Virulence Genes Detection of Aeromonas hydrophila Originated from Diseased Freshwater Fishes. Adv. Environ. Biol., 9(22), 22-26.

Sambrook, J., Fritscgh, E.F., Mentiates 1989. Molecular coloning. A laboratory manual. Vol !., Cold spring Harbor Laboratory press, New York.

Sharma, I., Rabha, D., Das, S., Ningombam, D. 2015. Hemolytic activity and antibiotic resistance of Aeromonas sp. isolated from marketed fish. Eur. J. Pharm. Med. Res., 2 (4), 304–312.

Singh, V., Rathore, G., Kapoor, D., Mishra, B.N., Lakra, W.S. 2008. Detection of aerolysin gene in Aeromonas hydrophila isolated from fish and pond water. Indian J. Microbiol. 48:453–458.

Sreedharan, K., Philip R., Singh, I.S.B., 2012. Virulence Potential and Antibiotic Susceptibility Pattern of Motile

Aeromonads Associated with Freshwater Ornamental Fish Culture System: A Possible Threat to Public Health. Brazilian Journal of Microbiology, 754-765.

Tomas J.M. 2012. The main Aeromonas pathogenic factors. ISRN Microbiol., 256261–256222. https://doi.org/10.5402/2012/256261.

Ullmann, D., Krause, G., Knabner, D., Weber, H., Beutin, L., 2005. Isolation and characterization of potentially
Journal of Biotechnology and Biochemistry, 4 (1): 51-53.

Yousr, A.H., Napis S., Rusul G.R.A., Radu, R. 2007. Detection of Aerolysin and Hemolysin Genes in Aeromonas spp. Isolated from Environmental and Shellfish Sources by Polymerase Chain Reaction. ASEAN Food Journal, 14(2): 115-122.

human pathogenic, cytotoxin producing Aeromonas strains from retailed seafood in Berlin, Germany. J. Vet. Med. Infect. Dis. Vet. Public Health, 52(2): 82-87.

Yogananth, N., Bhakyaraj, R., Chanthuru, A., Anbalagan, T., Mullai N.K. 2009. Detection of Virulence Gene in Aeromonas hydrophila Isolated from Fish Samples Using PCR Technique. Global.