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Molecular Characterization, Antimicrobial Susceptibility and Salt Tolerance of *Aeromonas hydrophila* from Fresh, Brackish and Marine fishes

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A.hydrophila;
Fresh;
Brackish;
Marine fishes;
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ABSTRACT

A total number of 170 fishes (100 fresh water, 40 brackish water, and 30 marine water fishes) from different farms in Alexandria, Kafr Elsheikh, and El-Behera governorates were collected from different water salinities. The prevalence of *Aeromonas hydrophila* was 47% (38% in fresh water fish, 65% brackish water fish, 53.3% in marine water fish). *A.hydrophila* isolates were tested for pathogenicity by inoculation into blood agar and skimmed milk agar 1% for hemolytic and proteolytic activity.
All isolates showed β hemolysis and proteolytic activity. *A.hydrophila* isolates were inoculated into trypticase soya broth with different NaCl concentration (0.5, 1, 2, 3, 4, 5, and 6%) and turbidity was measured by photometer at wave length 610 nm. The higher NaCl concentration, the lower turbidity was found. Moreover, reduction % of total bacterial count of *A.hydrophila* in relation to different concentration of NaCl in tryptic soya broth (TSB) after 10 fold serial dilutions by surface plating technique was calculated. Hemolysin and aerolysin genes were detected by PCR from the isolates obtained from different sources. *A.hydrophila* isolates from fresh, brackish and marine fish showed high resistance to Ampicillin (AM10), Erythromycin (E15) Nalidixic acid (NA30), and Spectinomycin (SPT10). In contrast, they showed high sensitivity to Enrofloxacin (EF10), Ofloxacin (OFX5) and Gentamicin (CN10). Polymixin (PB300 u) showed high activity against *A.hydrophila* isolated from brackish and marine fishes only. Doxycycline (DO30) and Nitrofurantoin (F300u) were highly effective against brackish water isolates than others for the field application. Isolation of *A.hydrophila* from different water salinities raises the public health concern and the importance to find suitable methods to control the infection.

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1. INTRODUCTION

A.hydrophila is a Gram negative, an opportunistic and zoonatically important primary fish pathogen which is the causative agent of bacterial hemorrhagic septicemia (motile aeromonad septicemia) (Chu and Lu, 2005; Austin and Austin, 2007). The disease is often associated with serious damage and economic losses in fish farming industry (Paniagua et al., 1990; Wang et al., 2003 and Sağlam et al., 2006). *A.hydrophila* produces several extracellular products such as proteases, haemolysins, aerolysin, cytolytic enterotoxins that are related with its pathogenicity (virulence) (Kingombe et al., 2010 and Hu et al., 2012). Secreted extracellular hemolysin and cytolytic enterotoxin by bacteria are reported to be important for causing certain lytic activities in host cells (Watanabe et al., 2004; Uma et al., 2010). The involvement of some virulence factors in Aeromonas species which was encoded by several genes has been demonstrated (Chacon et al., 2003 and Xia et al., 2004).
In the past, traditional microbiological and biochemical studies indicated that hemolytic and lytic activities were occurred in virulent *A. hydrophila* strains (Kozaki et al., 1987; Santos et al., 1988). As a common method, polymerase chain reaction (PCR) is used to identify Aeromonas genomospecies (Cascon et al., 1996; Khan and Cerniglia, 1997).
The main goals of the present study based on isolation and identification of *A.hydrophila* from fresh, brackish, and marine water fishes in Egypt. Moreover, determination of the salt tolerance of *A. hydrophila* from different isolates on selective media. In addition,

the sensitivity of different *A. hydrophila* isolates to commercial antimicrobial agents.

2. MATERIALS AND METHODS

2.1. Fish sampling

A total number of 170 fishes (100 fresh water fishes, 40 brackish water fishes, and 30 marine water fishes) were collected randomly from different fish farms in Alexandria, Kafr Elsheikh, and El-Behera governorates. All samples were taken directly to the lab for microbiological examination. Fish were examined clinically, for any abnormalities including hemorrhages, skin ulceration, fin erosions, abdominal distension. Bacteriological isolation of Aeromonas species were done from gills, liver, kidneys, spleen, heart, and muscles under aseptic conditions.

2.2. Isolation and identification of *A.hydrophila* in collected samples

Collected samples were directly inoculated into alkaline peptone water at 37 °C for 18-24 hours, and then cultivated onto Aeromonas agar plates, R.S medium, TCBS medium at 37 °C for 24 hours (Saglam et al., 2006). Suspected colonies were picked up and purified. All purified colonies were streaked on nutrient agar slopes and then preserved into semisolid nutrient agar for further identification**.**

Pure culture isolates were subjected to identification based on cultures characters, microscopic morphology and biochemical characterization according to Baumann and Baumann (1981) and the established methodologies of Austin and Austin (2007). The biochemical reactions used were; oxidase, catalase, indol, oxidation-fermentation (OF), Simmon"s citrate utilization, triple sugar iron (TSI), gelatin hydrolysis, and Aesculin hydrolysis.

2.3 Assessing the hemolytic and proteolytic activities of collected isolates

The collected isolates were examined for their hemolytic activity on sheep blood agar medium. Results were recorded after 24 hours of incubation at 37°C. The proteolytic activity of collected isolates was assayed using skim milk agar (nutrient agar containing 1% skimmed milk). The isolates were initially cultured into nutrient broth at 37 °C for 18-24 hours, and then 2 µl of broth were dropped on skim milk agar plates. The proteolytic activity was recorded according to the clear zone around the colony after 24 hour of incubation at 37°C (Takahashi et al., 2014).

2.4. Assessing the viability of collected isolates of *A.hydrophila* in increasing concentrations of NaCl by plate count technique

The growth of collected isolates of *A.hydrophila* was examined at different concentrations of NaCl (0.5, 1, 2, 3, 4, 5 and 6 %) in nutrient broth. The inoculated cultures were incubated at 37°C for18-24 hours. The growth turbidity was measured by colorimetric at OD 610 nm and the growth reduction % was calculated. Total viable count of bacterial isolates at different concentrations of NaCl (2, 4, 5 and 6%) was determined by surface count and pour plate methods as previously described by (Pianetti et al., 2008).

2.5. Detection of hemolysin and aerolysin genes in collected isolates by PCR

DNA extraction was performed by boiling method (Sambrook et al. 1989). Briefly, presumptive colonies were cultured in 6 ml tubes containing Aeromonas broth and incubated at 37°C for 24-48 h. The tubes were centrifuged at 10,000 rpm for 2 min. Harvested bacterial cells were washed with 500 µl of sterile distilled water and vortexed to re-suspend the pellets. Tubes were boiled at 100°C for 10 min and allowed to cool at -20°C for 5 min, followed by a centrifugation at 12, 000 rpm for another 5 min. The clear supernatant was ready to be used as template DNA in PCR assay. The PCR assay was carried out in 0.5 ml micro centrifuge tubes, with 20.0 µl of reaction mixture consisting of 11.9 µl sterile distilled water, 2.0 µl 10x PCR buffer, 1.6 µl 25 mM $MgCl_2$, 0.8 µl 2.5 mM deoxyribonucleotide phosphate (dNTP), 10 pmol of each of primers shown in table (1), 0.5 U of Taq

DNA polymerase, 1.2 µl of template DNA (Tanekhy et al., 2009). The PCR products were analyzed in 1.5 % agarose gel stained with ethidium bromide (10 mg/ml) and observed under UV Transilluminator using Gel Documentation System (Alpha Innotech, California, USA). The products were identified in comparison with the 100 bp DNA ladder (Real Biotech Corporation, Ohio, USA). Patterns of DNA fragments were compared.

3. RESULTS AND DISCUSSION

The severity of motile Aeromonas septicemia varies from species to another from hemorrhages, erosion, ulcers, high MR, congestion of internal organs as shown in Fig. 1 and Table 2. These finding are nearly similar to that obtained by Cagatay and Sen (2014). It was found that the total prevalence of bacterial infection was 47% which is higher than that recorded by Salama (1999) who found 36% in Nile tilapia but it is lower than that obtained by Ali (1994) who found 76.5% in tilapia. This may be attributed to the species and different water salinities, number of examined fishes and environmental conditions.

Table (1): Oligonucleotide primers sequences used in detection of *A.hydrophila*

Gene	Name	Sequence (5′-3′)	Product size (bp)
Hemolysin	Hly-F.	CACAGCCAATATGTCGGTGAAG	326
	Hyl-R.	GTCACCTTCTCGCTCAGGC	
Aerolysin	AHAF2. F.	GTGGCAAATAAGCGGTCTGG	209
	AHAR2. R.	CGATCAGACTGGGTTACATC	

Table (2): The prevalence of *A.hydrophila* isolated from different fish species.

Type	Fresh			Brackish				Marine				Total
species	Nile Tilapia	Common Carp	total	Catfish	Tilapia Zilli	Mugil cephalus	total	Morgan	Mugil capito	Yellow tail	total	
samples	80	20	100	10	14	16	40	8	12	10	30	170
positive	30	8	38	6	12	8	26	4	8	4	16	80
percentage	37.5	40	38	60	85	50	65	50	66	40	53.3	47

Fig. (1): Different fish species suffering from Aeromonas septicemic lesions. .A; catfish has severe hemorrhages at ventral aspect, B; Morgan has hemorrhage at operculum. C; N. tilapia has hemorrhages and scale loss, D and F; M. cephalus has hemorrhages and some dropsy. E; T.zilli has hemorrhage at fins. G; M. capito has hemorrhage at vent, H; Yellow tail has hemorrhage at operculum, I; hepatomegaly and splenomegaly in N. tilapia

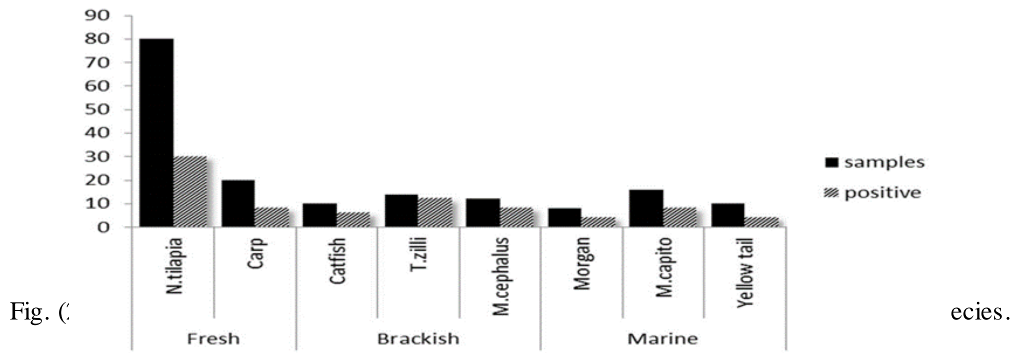

Fig. (: ... ecies.

In the present study, collected isolates of *A.hydrophila* were able to produce aerolysin and aerolysin-like hemolysin (ALH) as shown in Fig. (3). It also produced extracellular serine protease and metalloprotease and the properties of these proteases have been studied. These results confirmed that *A.hydrophila* produced an adequate amount of hemolysin to cause the accumulation of fluid, and its hemolysin plays an important role in the enterotoxicity. *A. hydrophila* isolates digested the skimmed milk around bacteria and formed a transparent zone. These results confirmed that *A.hydrophila* exerts the pathogenicity by many virulent factors as reported by Takahashi et al., (2014).

The physiological adaptation of bacterial cells to high NaCl concentrations has been associated with the modification of the circulation of sodium across the membrane (Padan and Krulwich, 2000). Furthermore, the synthesis of osmoprotectors such as betaine and proline, allows bacteria to tolerate osmotic stress (Wood et al., 2001). The obtained results indicated that the rate of growth of *A.hydrophila* was decreased as the NaCl concentration increased as indicated by the optical density readings as shown in Table 3 and 4. Indeed, it has been demonstrated that stressed and starved Aeromonas can enter a non-culturable state, most likely due to sublethal-injury mechanisms, including, among others, damage to the cell membrane (Mary et al., 2002). This state could explain the lower numbers of CFU ml^{-1} in comparison with viable cells observed when stressed bacteria were inoculated in fresh medium.

Fig. (3): showing noticeable hemolytic and proteolytic activities of *A.hydrophila*

Table (3): Results of bacterial growth turbidity in TSB using colorimetric at OD 610 nm.

NaCl	Normal	0.5%	1%	2%	3%	4%	5%	6%
Fresh	1.28	0.87	0.87	0.66	0.2	0.16	0.11	0.01
Brackish	1.45	1.12	0.96	0.83	0.24	0.21	0.16	0.02
Marine	1.5	1.5	1.18	1.01	0.26	0.24	0. 2	0.09

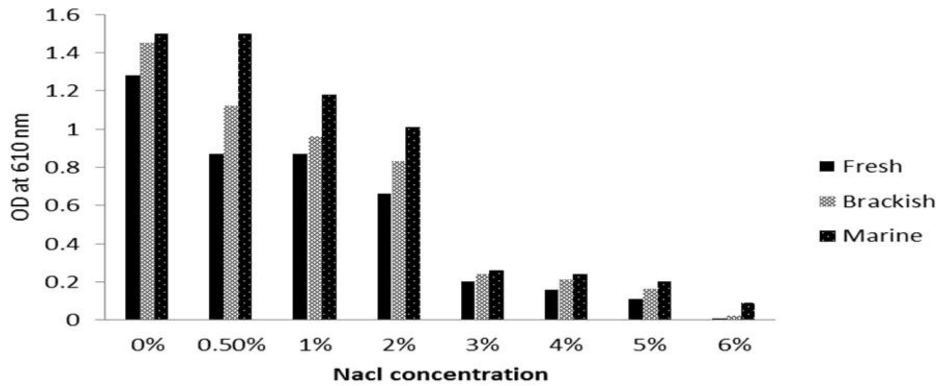

Fig.(4): Bacterial growth turbidity in relation to different salt concentrations.

Table (4): Reduction % of total bacterial count of *A.hydrophila* in relation to different concentration of NaCl.

	Fresh water	Brackish water	Marine water
Control (normal NaCl)	0%	0%	0%
NaCl (2%)	20%	6%	1.5%
NaCl (4%)	29%	4%	1%
NaCl (5%)	40%	38%	38%
NaCl (6%)	100%	100%	100%

The results of PCR for detection of *A.hydrophila* isolates that gave positive results by ordinary and biochemical tests, gave positive results with PCR for hemolysin and aerolysin genes (Fig. 5). Results confirmed that those primers were able to amplify two separate virulence genes in the 3 types of isolates. PCR amplification results of hemolysin and aerolysin genes from each group could indicate only virulent *A.hydrophila* (Cagatay and Şen, 2014). These genes were unable to detect nonpathogenic *A. hydrophila* strains, which did not contain the cytotoxin and hemolysine genes.

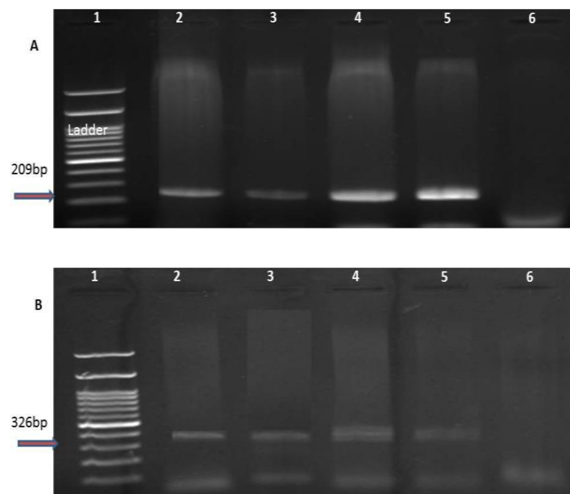

Fig. (5). Results of agarose gel electrophoresis of PCR product of Hemolysin and aerolysin genes of *A.hydrophila* isolated from fresh, brackish and marine fish. A: Hemolysin gene and B: Aerolysin gene where Lane (1) is 100 bp Ladder (DNA MW marker). Lane 2 is control positive, Lane 3 is Marine isolate, Lane 4 is brackish isolate, Lane 5 is Fresh isolate and Lane 6 is control negative.

Methods used to control Vibrio bacteria in aquaculture production systems include antibiotics and medicated feeds. Increasing antibiotic resistance poses important risks to human health (Musa et al., 2008) and can affect the course of infectious diseases (WHO, 1999). Results of *in vitro* antibacterial sensitivity of randomly selected 6 isolates of *A. hydrophila* from fresh, brackish, marine water fish to 15 antibiotics by disc diffusion was illustrated in table (5). *A.hydrophila* isolates from fresh, brackish and marine fish showed high resistance to Ampicillin (M10), Erythromycin (E15), Nalidixic acid (NA30) and Spectinomycin (SPT10). On the other hand, *A.hydrophila* isolates from fresh, brackish and marine fish showed high sensitivity to Gentamicin (CN10), Enrofloxacin (EF10), and Ofloxacin (OFX5). Polymixin (PB300u) showed activity against *A.hydrophila* from brackish and marine fishes only. Doxycycline (DO30), and Nitrofurantoin (F300u) were highly effective against brackish water isolates than others.

On conclusion, the present study represents the presence of potentially pathogenic *A. hydrophila* in different waters, and can infect various types of fishes including fresh, brackish and marine water fishes. *A.hydrophila* was found to have a variable resistance to some of widely used antibiotics. The public health significance of this microorganism should be monitored especially with their wide spread plus the genetic variation of this species in different water salinities.

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Table(5): Antibiogram sensitivity of *A.hydrophila* isolates from fresh, brackish and marine water fishes.

Antibiotic agent	*A.hydrophila* from Fresh water fish (n=6)						*A.hydrophila* from brackish water fish (n=6)						*A.hydrophila* from Marine water fish (n=6)					
	S		I		R		S		I		R		S		I		R	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Ampicillin(M10µg)	-	-	-	-	6	100	-	-	-	-	6	100	-	-	-	-	6	100
Erythromycin(E15 µg)	-	-	-	-	6	100	-	-	-	-	6	100	-	-	-	-	6	100
Nalidixic acid (NA30 µg)	-	-	-	-	6	100	-	-	-	-	6	100	-	-	-	-	6	100
Spectinomycin (SPT10 µg)	-	-	-	-	6	100	-	-	-	-	6	100	-	-	-	-	6	100
Polymixin (PB300u)	-	-	4	66	2	33	4	66	2	33	-	-	4	66	2	33	-	-
Colistin (CT10 µg)	2	33	-	-	4	66	6	100	-	-	-	-	4	66	-	-	2	33
Chloramphenicol (C30 µg)	2	33	2	33	2	33	2	33	4	66	-	-	2	33	-	-	4	66
Thiampinicol(TP30 µ)	4	66	-	-	2	33	4	66	2	33	-	-	4	66	-	-	2	33
Doxycycline(DO30 µg)	2	33	2	33	2	33	6	100	-	-	-	-	2	33	2	33	2	33
Tetracycline(TE30 µg)	-	-	-	-	6	100	4	66	-	-	2	33	2	33	-	-	4	66
Nitrofurantoin(F300u)	4	66	-	-	2	33	6	100	-	-	-	-	2	33	2	33	2	33
Sulphamethoxazole-Trimetroprim(SXT25 µg)	2	33	2	33	2	33	4	66	-	-	2	33	-	-	2	33	4	66
Cefotaxime(CTX30 µg)	-	-	4	66	2	33	-	-	-	-	6	100	-	-	2	33	4	66
Gentamicin(CN10 µg)	6	100	-	-	-	-	6	100	-	-	-	-	4	66	-	-	2	33
Enrofloxacin(EF10 µg)	6	100	-	-	-	-	4	66	-	-	2	33	6	100	-	-	-	-
Ofloxacin(OFX5 µg)	4	66	2	33	-	-	6	100	-	-	-	-	6	100	-	-	-	-

R: sensitive. I: Intermediate sensitive. R: resistant

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