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Investigation of some Virulence Determents in *Aeromonas hydrophila* Strains Obtained from Different Polluted Aquatic Environments

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Abstract

This study aimed to investigate some virulence characteristics of *Aeromonas hydrophila* (*A. hydrophila*) strains obtained from different Egyptian aquatic environments in terms of enzymatic activities, presence of virulence genes and their pathogenicity in *Oreochromis niloticus*. A total of 35 *A. hydrophila* isolates obtained from apparently healthy fish and water samples were examined. Isolates were collected from three different water sources of the River Nile; 15 isolates from El-Sharkawia stream that receives lofty loads of industrial effluents. 11 isolates from El-Rayah El-Towfeky stream at an area known to receive loads of sewage and agricultural discharges and 9 isolates from Bahr Yousuf canal at El-Fayoum governorate. Water and fish samples obtained from the studied areas were also analysed for existence of heavy metals. Isolates obtained from El-Sharkawia stream, exhibited the uppermost enzymatic activities and virulence genes compared to isolates retrieved from other sources. All *A. hydrophila* isolates were pathogenic to *Oreochromis niloticus.* Presence of heavy metal pollutants (Cr, Pb and Mn) in the aquatic environment affected the virulence of *A. hydrophila* isolates with variations in their enzymatic activities and presence of virulence genes.

Key words: *Aeromonas hydrophila*, Virulence, Aquatic Pollution**.**

1. Introduction

Bacteria of the genus *Aeromonas* are distributed widely in the aquatic environments. They have been commonly isolated in greater numbers from; rivers, lakes, streams, canals, sediments, marine as well as chlorinated water especially during hot months (Janda and Abbott, 2010). Certain *Aeromonas* strains have long been considered as serious pathogens in poikilothermic animals, including fish, amphibians and reptiles (Roberts, 2012). These microorganisms also are known to be causative agents of various infections in birds and mammals (Glunder and Siegmann, 1989). Array of human infections relevant to these pathogens also have been described, including gastroenteritis, urinary tract infections, pneumonia, wound infections and septicemia (Chan *et al*., 2003). On the top list of Aeromonads species affecting aquatic animals, *A. hydrophila* has long been considered a pathogen of critical concern in commercial aquaculture causing colossal economic losses (Elgendy *et al*., 2015). Wide spectrum of fresh as well as marine fish species can be common targets for such epizootics (Moustafa *et al*., 2010; Austin and Austin, 2012). As an opportunistic pathogen, fish diseases caused by *A. hydrophila* are linked to unfavorable environmental conditions (Elgendy *et al*., 2015). Pollutants stemming from anthropogenic activities are among the notorious sources of stress conditions overwhelming aquatic species predisposing them to array of microbial infections (Moustafa *et al*., 2015). These pollutants may affect aquatic animals either directly via suppression of immune defense mechanisms or potentially through their effect on the virulence determents of attacking pathogens (Arkoosh *et al*., 1988). Heavy metal pollution affects lysozyme levels and causes alterations of immunoregulatory functions in fish (Sanchez-Dardon *et al*., 1999). Heavy metals also can compromise fish humoral and cell mediated immunity through many pathways, including decreasing levels of antibody and splenic plaque forming cells, reducing proliferation of splenic lymphocytes, and via decreasing disease resistance to bacterial infection (Khangarot *et al*., 1999). The

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pathogenicity of *A. hydrophila* has been ascribed to numerous virulence determents. A number of virulence factors, including secretion systems, adhesins , toxins, enzymes, quorum systems, iron acquisition and antibiotic resistance, have been identified (Tomas, 2012). In this study, we investigated the presence of six virulence genes (*Act*, *Ast*, *Lip*, *Elast*, *Alt* and *Fla*) in *A. hydrophila* strains obtained from aquatic environments. In addition, the virulence of *A. hydrophila* strains was tested by experimental infection in *O. niloticus*.

2. Material and Methods

2.1. Bacterial Strains and Sampling

A total of 35 *A. hydrophila* isolates obtained from apparently healthy fish and water samples were analyzed in the present study. Isolates were obtained from three water sources; 15 isolates (10 isolates from water and 5 isolates from fish samples) from El-Sharkawia stream (a narrow tributary of the River Nile between Shubra El kheima and El Qanater El Khayreya region, Qaluobyia) which receives lofty loads of industrial effluents coming from numerous factories in that region, like textile, paper, plastic, oil, soap, detergents and small scale services (like petrol stations and garages). 11 isolates (7 isolates from water and 4 isolates from fish) from El-Rayah El-Towfeky stream at Barshoom that receives loads of sewage and agricultural effluents. Additionally, 9 isolates were retrieved from Bahr Yousuf canal at El-Fayoum governorate, a great canal linking the main branch of the River Nile to provide water permanently to the Lake Qarun at El-Fayoum governorate, (5 isolates from water and 4 isolates from fish).

All samples were cultured onto Aeromonas agar base supplemented with Ampicillin, to select for green colonies. All isolates were confirmed with, the commercial Api 20-NE.

2.2. Hemolysin, Protease, Gelatinase and Slime Production

Haemolysis was assayed by spreading each strain onto 5% rabbit erythrocytes agar plates for 24 h at 30°C. Protease activity was demonstrated by spreading *Aeromonas* strains on nutrient agar containing 1.5% skim milk. Plates were incubated for up to 72 h at 30°C, the production of protease was shown by the formation of a clear zone. Gelatinase production was determined using Luria Broth agar containing gelatin (30 g L^{-1}), the plates were incubated overnight at 30°C and then cooled for 5 h at 4°C. The appearance of a turbid halo around the colonies was considered positive for gelatinase production according to Sechi *et al*. (2002).

Slimness was investigated by culturing bacterial isolates onto Brain Heart infusion agar containing 0.8 g l^{-1} Congo Red (Sigma-Aldrich, Milan, Italy). Plates were inoculated at 30°C for 24 h. Slime producing bacteria appeared as black colonies whereas non-slime producers remained non pigmented (Freeman *et al*., 1989).

2.3. PCR for Detection of Virulence Genes

Genomic DNA was extracted from bacterial isolates using DNA extraction kit (Fermentas, Lithuania) according to manufacturer's instructions. The primer pairs used for PCR amplification of six virulence genes (shown in Table 1): cytotoxic heat-labile enterotoxin (Act), cytotonic heat-stable enterotoxin (Ast), Lipase (Lip), elastase (Ela), cytotonic heat-labile enterotoxin (Alt) and flagella A as well as flagella B (Fla), using the same primers sequences and PCR conditions as reported by (Sen and Rodgers, 2004). Briefly, reactions were performed in 25µl volumes, each contained 1 µM of each primer, 12.5 µM of AmpliTaq™ Gold PCR Master mix (2X) containing, $MgCl_2$, AmpliTaq™ Gold DNA polymerase, and dNTPs (Applied Biosystems). 80 ng of DNA template in 5 µl volume was used. Cycling conditions consisted of an initial single cycle at 95°C for 5 min, followed by 25 cycles of 25 s at 95 °C, annealing for 30 s at 55 °C, elongation for 1 min at 72 °C and a final single elongation cycle at 70 °C for 5 min. PCR was performed in Eppendorf Master Cycler gradient thermocycler (Eppendorf AG, Hamburg, Germany).

Table 1. Set of primers used for amplification of virulence genes

Gen	Primer sequence	Size of product bp	Reference
Act	F 5-GAAGGTGACCACCAAG AACA-3 R5-AACTGACATCGGCCTTGAACTC-3	232	Kingombe *et al.,* (1999)
Ast	F5-TCTCCATGCTTCCCTTCCACT-3 R5-TGTAGGGATTGAAGAAGCCG-3	331	Sen and Rodgers (2004)
Fla	F5-TCCAACCGTYTGACCTC-3 R5-GMYTGGTTGCGRATGGT-3	608	Sen and Rodgers (2004)
Alt	F5-TGACCCAGTCCTGGCACGGC-3 R5-GGTGATCGATCACCACCAGC-3	442	Sen and Rodgers (2004)
Lipase	F5-ATCTTCTCCGACTGGTTCGG -3 R5-CCGTGCCAGGACTGGGTCTT-3	382	Sen and Rodgers (2004)
Elastase (Ela)	F5-ACACGGTCAAGGAGATCAAC-3 R5-CGCTGGTGTTGGCCAGCAGG-3	513	Sen and Rodgers (2004)

2.4. Virulence Test

The artificial infection of 12 *A. hydrophila* strains (4 isolates from each site) was carried out by injection in *Oreochromis niloticus* (Table 2). 130 Fish weighing between 40- 50 g were used in the present study. Fish were kept in glass aquaria (60×30×40 cm^3) at a rate of 10 fish / aquarium. The fish were maintained at 27°C and fed with a commercial diet twice a day. Compressed air was pumped continuously into the feed tanks and 50% water was exchanged every day. All bacterial strains were cultured in tryptic soy broth for 24 h at 27°C. All bacterial strains were adjusted to 1 x 10^7 Colony forming Unit (CFU)/ml in Phosphate Buffer Saline. Fish were intraperitoneally injected with 0.2 ml of each bacterial culture while the control group was injected with 0.2 ml Phosphate Buffer Saline and kept under observation for 10 days according to Abu-Elala and Ragaa (2015).

Table 2. Virulence factors of *Aeromonas hydrophila* isolates

source	strain	Haemolysis	Gelatinase	Protease	Slime test	PCR detection of virulence gens					
						ACT	AST	LIP	ELAST	ALT	FLAG
El-Sharkwia stream	1 w *	+	-	+	-	-	-	-	-	-	-
	2 w	-	-	+	-	+	-	-	-	-	-
	3 w	+	-	-	-	-	-	-	-	+	-
	4 w *	+	-	-	-	+	-	+	-	-	-
	5 w	-	-	-	+	-	-	-	+	-	-
	6 w	-	-	+	+	+	-	-	+	-	-
	7 w	-	-	-	-	-	-	-	-	+	-
	8 w	+	-	-	-	-	-	-	+	-	-
	9 w	+	-	-	-	-	-	+	-	-	-
	10 w*	-	-	-	-	-	-	+	-	-	-
	11 f	+	-	+	-	+	-	-	+	-	-
	12 f	+	-	+	+	-	-	-	+	-	-
	13 f	-	+	-	+	+	-	+	-	+	-
	14 f	+	+	-	-	-	-	-	+	-	-
	15 f *	+	-	+	+	+	-	+	-	-	-
El-Rayah El-Towfeky stream	16 w	-	-	-	-	-	-	-	+	-	-
	17 w *	+	-	-	-	-	-	-	-	-	-
	18 w	-	-	-	-	-	-	+	+	-	-
	19 w	-	-	-	+	-	-	-	-	+	-
	20 w *	+	+	+	-	+	-	-	-	-	-
	21 w	-	+	-	-	-	-	-	-	-	-
	22 w*	-	-	-	-	-	-	-	-	-	-
	23 f *	+	+	+	+	-	-	-	-	-	-
	24 f	-	+	-	-	-	-	-	+	-	-
	25 f	-	-	-	-	+	-	+	+	+	-
	26 f	-	-	-	+	-	-	-	-	-	-
Bahr Yousuf at El-Fayoum governorate	27 w *	-	-	-	-	-	-	-	-	-	-
	28 w	-	-	-	-	-	-	-	-	-	-
	29 w	-	-	-	-	-	-	-	-	-	-
	30 w	-	-	+	-	-	-	-	-	-	-
	31 w	-	-	-	-	+	-	-	-	-	-
	32 f *	-	-	-	+	-	-	-	+	-	-
	33 f	-	-	+	-	-	-	-	-	-	-
	34 f *	-	-	-	-	-	-	-	+	-	-
	35 f *	+	-	+	+	-	-	-	-	+	-

*isolates used in pathogenicity testing; W= water; F= fish

2.5. Total Aerobic Count and Total Coliform Count

Bacterial counts were carried out in triplicate. Twenty five gram of fish muscles (5 fish per site) was aseptically cut and transferred into sterile stomacher bag containing 225 ml sterile maximum recovery diluents (Oxoid) according to APHA (1992). Similarly, 25 ml of each water sample (3 samples per site) was mixed with 225 ml of the same recovery diluents. Decimal dilution was prepared up to 10^{-6} and Pour plates were prepared from l0 fold dilutions in plate count agar (Oxoid) for total aerobic enumeration, and violet red bile agar (VRBLA, Oxoid) for total coliforms. All counts were made after incubation of all plates at 35°C for 24 h. Bacterial colonies were counted and expressed in Colony forming Units (CFU) per gram of fish muscles and as (CFU /mL) for water samples (Collins and Lyne, 1984).

2.6. Physiochemical Water Analysis

Temperature, pH and dissolved oxygen (DO_2) were measured on spot at each collection site by digital apparatus, YSI (Yellow Springs, Ohio USA). Chemical Oxygen Demand (COD) was carried out using the potassium permanganate method (Golterman, 1971) and biochemical oxygen demand (BOD) with the five-day incubation method (APHA, 2000). Nitrates (NO_3) and ammonia (NH_3), in water samples were assessed according to methods adopted from (APHA, 2000). Regarding heavy metals, water samples were acidified by concentrated nitric acid (5 mL/L) and then heavy metals (Cd, Co, Cr, Cu, Pb, Ni, Zn and Mn) were measured by atomic absorption spectrophotometer (Perkin-Elmer 3110, USA) (APHA, 2000). Pollution with organochlorines and organophosphates was also analyzed quantitatively using an Agilent gas chromatograph 6890 (Dahshan *et al*., 2016).

2.7. Heavy Metal Analysis in Fish Tissues

Fish were dissected; muscles and liver tissues were isolated, weighed, put in glass vials and digested in concentrated nitric acid (Merck, Darmstadt, Germany); then placed on a hot plate at 100 °C. Samples were cooled in room temperature after complete digestion then, filtered and reached a volume of 10 ml by distilled water. Heavy metal concentrations (Cd, Co, Cr, Cu, Pb, Ni, Zn and Mn) were determined by atomic absorption spectrophotometry

(Perkin-Elmer 3110, USA) (APHA, 2000). Accumulation Factor (AF) was calculated according to Authman *et al*. (2013).

3. Results

3.1. Hemolysin, Protease, Gelatinase Production and Slime Test

37.14 % of *A. hydrophila* strains were able to lyse rabbit erythrocytes and produced haemolysis on rabbit blood agar plates. 31.42 % of isolates produced protease and hydrolysed Skimmed milk, 17.14 % were gelatinase positive, 28.57 % were positive in slime production test (Table 2).

3.2. PCR for Detection of Virulence Genes

All isolates were found negative for the virulence genes, *Ast* and *Fla* whereas nine strains produced the expected band, 232 bp of *Act*. Seven strains showed a positive band of 382 bp respective for *Lip*. Twelve strains gave the expected band at 513 bp of *Elast*. Six isolates were positive for *Alt* showing expected band at 442 bp (Table 2; Figure 1).

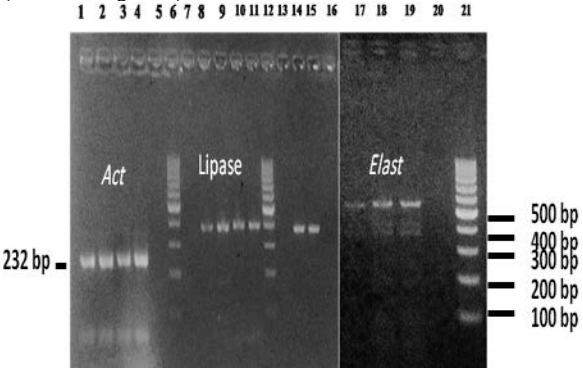

Figure 1: Agarose gel electrophoresis. Lanes 6, 12 and 21: 100 bp DNA marker. Lanes 1,2,3, 4: *Act* positive strains; Lanes 8, 9, 10, 11, 14, 15 are Lipase positive strains; Lanes 17, 18, and 19 are Elastase positive strains; Lanes 5, 7, 13, 20 Negative strains

3.3. Virulence Test

All experimentally infected fish, which were injected with the different *A. hydrophila* isolates (Table 2), died during the period of observation. *A. hydrophila* were re-isolated from the kidney of succumbed fish. Fish showed petechial hemorrhages on different parts of the external body surfaces. Internally, congestion of the liver and spleen were commonly detected. *A. hydrophila* were re-isolated from all succumbed fish. Fish injected with saline showed no mortalities.

3.4. Total Aerobic Count and Total Coliform Count

The mean total aerobic bacterial and total coliform counts in water and fish samples are illustrated in (Table 3). Counts were highest in samples collected from El-Rayah El-Towfeky stream since the total aerobic bacterial counts was $2.5X10^3$ CFU/mL and $1.6X10^4$ CFU/gm in water and fish samples, respectively, while the mean total coliform counts was $6X10^2$ CFU/mL and $4.3X10^3$ CFU/gm, respectively. The lowest bacterial counts were recorded in water and fish samples collected from El-Sharkawia stream; the total aerobic bacterial counts was $1.9X10^3$ CFU/mL and$7.5X10^3$ CFU/gm in water and fish samples while the mean Total coliform count was $2X10^2$ CFU/mL and $1.5X10^3$ CFU/gm, respectively.

Table 3. Total bacterial aerobic count and total coliform count

	Total aerobic count		Total coliform count	
	water	muscles	Water	muscles
El-Sharkawia stream	$1.9X10^3$ CFU/ml	$7.5X10^3$ CFU/gm	$2X10^2$ CFU/ml	$1.5X10^3$ CFU/gm
El-Rayah El-Towfeky	$2.5X10^3$ CFU/ml	$1.6X10^4$ CFU/gm	$6X10^2$ CFU/ml	$4.3X10^3$ CFU/gm
Bahr Yousuf at El-Fayoum governorate	$2.0X10^3$ CFU/ml	$3.5X10^4$ CFU/gm	$3X10^2$ CFU/ml	$2.5X10^3$ CFU/gm

3.5. Physiochemical Water Analysis

The average values recorded for water temperature, pH and dissolved oxygen were within normal values. BOD and COD values were slightly more than the permissible values, especially at El-Sharkawia stream; they were 12.43 and 17 mg/L, respectively. NO_3 and NH_3 were within the permissible limits. Concentrations of heavy metals demonstrated variable levels. Chromium (Cr), Lead (Pb) and Manganese (Mn) showed high values that exceed the permissible limits in El-Sharkawia stream; 1414, 67and 567 µg/L, respectively. Chromium is also the most determined metal in El-Rayah El-Towfeky (1192 µg/L), while Cu was not detected in both locations, and Cd, Ni and Zn were in the permissible limits. On the other hand, Co, Cr, Ni and Mn were not detected in water samples collected from Bahr Yousuf canal. At the same location Cd, Cu, Pb and Zn were within the permissible limits Pesticides; organochlorines and organophosphates were not detected in all investigated water samples (Table 4).

Table 4. Physicochemical water analysis

Permissible Limits		Bahr Yousuf	El-Rayah El-Towfeky stream	El-Sharkawia stream	
USEPA, 2004	Egyptian Environmental Law No.48, 1982				
		25±1.7	26±1.3	26±1.5	Temperature (^{o}C)
	6.5-8.5	8.3±0.6	7.5±0.4	6.9±0.5	pH
	<5	6.4±0.3	6.3±0.6	5.9±0.8	DO_2 (mg/l)
	<6-10	10.8±0.6	11.19±0.5	12.43±0.8	BOD (mg/l)
	<10-15	15±0.8	12±0.9	17±1	COD (mg/l)
	40	0.61±0.02	0.59±0.04	0.59±0.02	No_3 (mg/l)
	< 3	2.1±0.02	1.7±0.03	2.06±0.02	NH_3 (mg/l)
	Heavy metals (µg/l)				
0.25	10	8±0.4	2±0.3	1±0.5	Cd
-	-	nd	21±3	20±1	Co
74	50	nd	1192±70	1414±80	Cr
9	1000	31±1	nd	nd	Cu
2.5	50	28±1	36±0.9	67±1	Pb
52	100	nd	12±6	6±0.2	Ni
120	1000	66±9	11±5	19±3	Zn
-	500	nd	239±10	567±80	Mn
	Pesticides				
		nd	nd	nd	Organochlorines
		nd	nd	nd	Organophosphates

nd (not detected)

3.6. Metals Residues in Fish Tissues

The determined heavy metal concentrations and their bioaccumulation factors in liver tissue were more than that in muscle tissues. Heavy metals exhibited different values of accumulation in fish muscles in both El-Sharkawia and El-Rayah El-Towfeky while in Bahr Yousuf canal all the recorded metals were within the recommended permissible limits (Table 5).

Table 5. Heavy metals residues (mg/Kg wet weight) in muscles and livers of *Oreochromis niloticus* fish from the different studied localities

	El-Sharkawia stream		El-Rayah El-Towfeky stream		Bahr Yousuf at El-Fayoum governorate		Permissible limits	
	M	**L**	**M**	**L**	**M**	**L**	**FAO, 1983**	**IAEA, 407**
Cd	0.18±0.016 (180)	0.16±0.005 (160)	0.16±0.007 (80)	0.14±0.003 (70)	0.004±0.001 (0.5)	0.005±0.0003 (0.63)	0.05	0.189
Co	0.10±0.022 (5)	0.06±0.001 (3)	0.26±0.025 (12.38)	0.52±0.005 (24.76)	nd	nd	---	0.1
Cr	3.40±0.020 (2.40)	6.18±0.012 (4.37)	2.48±0.041 (2.08)	4.92±0.034 (4.13)	nd	nd	1.0	0.73
Cu	1.08±0.092 (--)	3.00±0.071 (--)	4.74±0.111 (--)	36.18±0.311 (--)	0.0003±0.0001 (0.01)	0.001±0.0002 (0.03)	30	3.28
Pb	0.14±0.007 (2.09)	0.24±0.005 (3.58)	0.28±0.033 (7.78)	0.34±0.012 (9.44)	0.009±0.001 (0.32)	0.02±0.002 (0.71)	0.5	0.12
Ni	1.34±0.013 (223.33)	3.06±0.034 (510)	1.66±0.007 (138.33)	3.60±0.014 (300)	0.21±0.003 (--)	0.37±0.012 (--)	---	0.6
Zn	11.44±0.034 (602.11)	24.22±0.36 (1274.74)	18.76±0.097 (1705.45)	21.06±0.29 (1914.55)	0.022±0.002 (0.33)	0.046±0.003 (0.70)	30	67.1
Mn	1.24±0.033 (2.19)	1.16±0.007 (2.05)	1.30±0.005 (5.44)	2.30±0.003 (9.62)	0.26±0.002 (--)	0.41±0.011 (--)	1.0	3.52

M: muscle, L: liver, nd: not detected; Data are represented as mean value of three replicates of pooling tissues ± Standard deviation (Accumulation factor).

4. Discussion

In the present study, virulence determents of *A. hydrophila* isolates are analysed by enzymatic and molecular methods. The present study demonstrates a difference in the enzymatic activities and extracellular products produced by *A. hydrophila* isolates obtained from different environmental sources. Isolates obtained from El-Sharkwia stream that receives high industrial effluents exhibited the uppermost enzymatic activities compared to isolates retrieved from other sources. *A. hydrophila* secretes a wide range of extracellular products, including proteases, lipases, nucleases, and gelatinases that potentiate their virulence as well as adaptation to environmental changes. 37.14 % of *A. hydrophila* strains were able to lyse rabbit erythrocytes; nine isolates from El-Sharkawia stream, three from El-Rayah El-Towfeky stream and only one isolate from Bahr Yousuf canal at El-Fayoum governorate. *Aeromonas* sp. produce two types of hemolysins without enterotoxic properties; α-hemolysins and β- hemolysins which are thermostable and pore forming toxins that lead to osmotic lysis and complete destruction of erythrocytes (Kirov, 1997). Proteases were also detected in some isolates (31.42 %) with high frequencies in strains obtained from El-Sharkawia stream (six isolates). Extracellular proteases allow *Aeromonas* sp. to persist in different habitats and facilitate ecological interactions with other organisms. Proteases enhance the establishment of infections and overcoming the initial host defenses (Leung and Stevenson, 1988). Additionally, proteases promote invasion by direct damage of host tissue or by proteolytic activation of toxins (Kirov, 1997). Gelatinase was completely absent in isolates obtained from the fish farm samples where it was detected in four isolates in El-Rayah stream that receive loads of sewage and agriculture effluents. Gelatinase contribute significantly in the pathogenicity as it enhances the intestinal colonization by bacteria through the disruption of the intestinal barrier.

PCR detection of virulence gens demonstrated absence of *Ast* and *Fla* in all isolates. Sen and Rodgers (2004) detected the genes coding for the polar flagellum protein in 59% of *Aeromonas* strains obtained from drinking water and they alleged that presence of flagella is critical for the adherence process of pathogens since mutations in the polar flagellum *flaA* and *flaB* genes resulted in complete loss of motility and adherence to human epithelial HEp-2 cells. Nine strains produced the expected band for PCR amplification of *Act*, six of these isolates were obtained from samples from El-Sharkawia stream. *Act* is one of the main virulence factors of *A. hydrophila* and have hemolytic, cytotoxic and enterotoxic activities (Tomas, 2012). This cytotoxic enterotoxin perform important roles in Aeromonas infections as it induces early cell signaling in eukaryotic cells, which leads to the production of inflammation mediators in macrophages (Xu *et al*., 1998) and also contributes to apoptosis (Galindo *et al*., 2006). Seven strains showed a positive band of *Lip* none of them from the fish farm. Lipases can affect several immune system functions through the free fatty acids generated by lipolytic activity. *Elast* was detected in twelve isolates while six isolates demonstrated positive results for *Alt*. *Alt* is a cytotonic enterotoxin which does not cause degeneration of crypts and villi of the intestine like the cytotoxic enterotoxin (Chopra and Houston, 1999). A significant link between existence of *alt* and *ast* genes in *Aeromonas* isolates and their ability to cause infections and diarrhea in children harboring such strains (Albert *et al.*, 2000). In their study, the authors detected that 54% of the *A. hydrophila* strains obtained from diarrheal children had both genes, while only 15% of the strains recovered from environmental samples were found to have both genes. Harboring of enterotoxins alone may not be sufficient for virulence since these factors have been found in strains isolated from healthy and the ability of the bacterium to adhere and invade the intestinal mucosa are also crucial components of pathogenesis (Schiavano *et al*., 1998). It has been demonstrated that, bacterial strains from environmental sources are significant reservoirs of virulence and fitness genes and acquisition of such genes occurs among autochthonous bacteria as well as human pathogens, released via anthropogenic activities, in the aquatic environment (Xie *et al*., 2005; Caburlotto *et al*., 2009). The acquisition of mobile genetic elements, such as plasmids, bacteriophages, transposons and genetic islands allows bacteria to acquire a range of genetic traits that may increase their fitness under different environmental conditions and their virulence potentials (Gennari *et al*., 2012).

Results did not show an obvious correlation between the presence of virulence determents in *A. hydrophila* isolates and pathogenicity to *Oreochromis niloticus*, as some of these strains possess more or less virulence determents. Additionally some of these strains No (22 w* and 27 w *) were pathogenic and resulted in death of all challenged fish, although they do not possess any of the studied virulence determents. This suggests that *A. hydrophila* might have a different virulence gene system and different pathogenic mechanisms other than the studied and further genotypic studies are still needed to elucidate the hypothesis.

Bacterial counts were higher in water collected from El-Rayah El-Towfeky stream which may be relevant to the surplus sewage and agricultures discharges. Consequently bacterial loads were higher in fish muscles samples collected from the same site since the level of contamination of aquatic animals depends on the extent of pollution in the growing waters (Ekanem and Adegoke, 1995). On the other hand the lowest bacterial counts were detected in Sharkawia stream which may be due to the toxic effects of industrial pollutants on the bacterial communities distributed in the area.

Comparing the physicochemical picture of the water in the three studied locations, El-Sharkawia stream was the most polluted source. It receives high industrial effluents and consequently high BOD and COD values. The water was loaded by high concentrations of some heavy metals (Cr, Pb and Mn) that exceed the Egyptian Environmental law no. 48, (1982) and international guidelines of freshwater of USEPA (2004). The lofty loads of chromium detected in El-Sharkawia and El-Rayah streams may be attributed to the industrial and agricultural discharges (Ahmed *et al*., 2013). The majority of the recorded metals were concentrated and accumulated in fish tissues to levels exceed the international permissible limits FAO, 1983 and IAEA, 2003 especially in liver due to its main detoxification function (Iwegbue, 2008). The variation in

the accumulation of metals in fish organs may be relevant to the availability of metals to specific fish tissues, fish age, species, the existence of ligands in the tissues with a high affinity to the metal and/or to the role of these tissues in the detoxification process (Bashir and Alhemm, 2015). On the other hand, Bahr Yousuf canal was the lowest polluted location, has nearly normal water quality parameters and all the detected metals were within the recommended permissible limits both in water and in tissue samples. The presence of heavy metals pollutants in the aquatic environment can result in deleterious impacts on the ecosystems, with alterations in the biomass, diversity of microbial communities and cycling of elements (Sobolev and Begonia, 2008). The toxic effects of heavy metals on microorganisms are influenced by many factors, such as pH, concentration of chelating agents and organic matter (Nwuche and Ugoji, 2008). Long existence of microorganisms in such polluted environments results in selection of certain strains of bacterial populations (Silva *et al*., 2012). Bacterial strains also can exhibit adaptation to heavy metals pollutants in their environment through; changing the metabolic and physiological activities of bacteria (Lima e Silva et *al*., 2012); formation of resistant strains as well as via altering the genetic information (Chudobova *et al*., 2015).

5. Conclusion

Environmental *A. hydrophila* strains have array of virulence attributes and isolates obtained from industrially polluted area demonstrated existence of the highest level of virulence characteristics. The presence of metallic pollutants in the aquatic environment can affect the enzymatic activities as well as the genetic virulence factors of cohabitant bacterial populations. Additionally, *A. hydrophila* retrieved from aquatic environment are highly pathogenic to fish and have diverse pathogenic mechanisms and further genotypic studies are still needed to elucidate the hypothesis.

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