Detection of some virulence genes in *A. hydrophila* and *A. caviae* isolated from fresh water fishes at Qalubia Governorate

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A B S T R A C T

The study was conducted on 225 diseased fish samples, 125 Nile tilapia (*Oreochromis niloticus*) and 100 Catfish(*Claris gariepinus*) , collected from different fish markets at Qalubia Governorate during the period from January (2016) to May (2017) for detection of Aeromonas species. The samples were taken from apparently pathognomonic lesions in muscle, kidney, liver, intestine and spleen after clinical and postmortem examination for bacteriological examination. The results revealed that, 125 Aeromonas species were isolated from the examined samples where *A. hydrophila* and *A. caviae* were identified. Accurately 114 (91.2 %) *A. hydrophila* strains, 63 (50.4%) and 51 (40.8%) were isolated from *C. gariepinus* and *O. niloticus* fishes respectively. Meanwhile, 11(8.8 %) *A. caviae* strains, 7 (5.6%) and 4 (3.2%) from *C. garicpinus* and *O. niloticus* fishes respectively. Further PCR results for virulence genes in isolated Aeromonas strains indicated that, *aero* gene was detected in 9 out of 10 *A .hydrophila* studied strains and in 3 out of 6 *A. caviae* ; *hly* gene in 7 out of 10 *A .hydrophila* and in 2 out of 6 *A. caviae*; *Ahcytoen* gene in 6 out of 10 *A .hydrophila* and in 1 out of 6 *A. caviae* ; *act* gene in 6 out of 10 *A .hydrophila* and in 3 out of 6 *A. caviae* and *ast* gene in 7 out of 10 *A .hydrophila* and in 3 out of 6 *A. caviae* studied strains. Finally, the production of a wide array of virulence factors by isolated strains is indicative of their potential to cause diseases in fishes and humans.

Key words: Fish, bacteriological, Aeromonas, virulence genes

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1. INTRODUCTION

Bacterial pathogens are the most serious disease affecting fish resulting in high mortalities and economic losses among fish and fish farms (Austin and Austin, 2007). Aeromonas species are responsible for wide range spectrum of diseases among fish and human, as Motile Aeromonas Septicemia (MAS) in fish which is caused by *A .hydrophila* leading to high mortalities and high economic losses (Ebanks *et al.*, 2005;Vivekanandhan *et al.*, 2005; and Shayo *et al.*, 2012).

The genus Aeromonas is a member of the family Aeromonadaceae. The genus has

undergone a number of nomenclatural revisions in recent years and there are now 30 recognized species in the genus Aeromonas. The most predominant species are *A. hydrophila*, *A. caviae* and *A. veronii* biotype *sobria*. They are Gram-negative rods, either straight or curved facultative anaerobes, catalase-positive and most are motile by polar flagella. All of them ferment glucose with acid production and a few species produce gas; they produce diastase, lipase, DNase and various proteinases and most of them will grow on common laboratory media at 35–37°C.

Gastrointestinal tract infections are the commonest source of Aeromonads followed by wound infections. In immunosuppressed individuals or those with hepatobiliary disease, aeromonads can cause otitis media, meningitis, endocarditis, peritonitis, cholecystitis, hemolytic uremic syndrome, septicemia and food poisoning (Ko *et al*., 2000 and Guerra *et al.*,2007).Moreover, the isolated *A. hydrophila* strains from patients with gastroenteritis are haemolytic (Wejdan *et al*., 2014).

Members of the Aeromonas, are Gram-negative rods (0.5–0.8 × 3.0–4.0 μm) which are either straight or curved. They are facultative anaerobes, catalase-positive and most are motile by polar flagella. Aeromonads produce extracellular enzymes (haemolysins, lipases, proteases, β-lactamases, amylases, chitinases and nucleases) involved in their ecology, survival and pathogenicity (Stratev *et al*., 2015). The pathogenicity of motile Aeromonads have been linked to some virulence factors produced by them including structural features associated with adhesion, cell invasion, resistance to phagocytosis as well as extracellular factors such as aerolysin, a pore-forming toxin, which is cytolytic and enterotoxin genie (Chopra and Houston 1999 and Rabaan *et al.,* 2001).

Haemolysins (haemolysin and aerolysin) belong to a large group of pore-forming bacterial cytolysins, which can cause cytoplasmic content leakage by breaking the cellular membrane, and ultimately, cell death (Heng *et al*., 2005 and Samal *et al*., 2014). Exotoxins are major virulence factors of aeromonads that include a cytotoxic heat-labile enterotoxin (*act*) resulted in extensive damage to epithelium as it possesses hemolytic and cytotoxic activities in addition to an enterotoxic activity, aerolysin/haemolysin; a cytotonic heat-labile enterotoxin (*alt*), lipase, extracellular lipase, or phospholipase and a cytotonic heat- stable enterotoxin (*ast*) (Bin Kingombe *et al.,* 2010).

As Aeromonas are considered one of the most important fish pathogens and can be a problem for human consumers and fish, had attained a great economic importance in Egypt, so, the present study was conducted to throw light over the Aeromonas infection in fresh water fish, bacteriological characterization of isolated strains and detection of some virulence genes of the them by using P C R .

2. Materials and methods

2.1. Samples collection:

Accurately, 225 diseased fish samples, 125 Nile tilapia (*Oreochromis niloticus*) and 100 Cat fish(*Claris gariepinus*) ,of various sizes were collected from different fish markets at Qalubia Governorate during the period from January (2016) to May (2017) for demonstration of Aeromonas strains.

2.2. Clinical and postmortem examinations were performed using the method described by Schaperdaus *et al*., (1992).

2.3. Bacteriological examination

2.3.1. Sampling:

After clinical and postmortem examination of collected fish samples, 432 samples collected from 225 diseased fishes; 240 samples from 125 Nile tilapia (*O. niloticus*) where the samples were collected from apparently pathognomonic lesions in muscle, kidney, liver, intestine and spleen by a number of 72, 55, 68, 36 and 9 respectively and 192 samples from 100 Catfish(*Claris gariepinus*), the samples were gathered from apparently pathognomonic lesions in muscle, kidney, liver, intestine and spleen by a number of 63, 41, 47,32 and 9 respectively.

2.3.2. Isolation and identification of suspected Aeromonas species:

The surface of lesions were seared by hot spatula, then a sterilized loopful was introduced through seared portion and inoculated onto Tryptone soya broth then incubated aerobically at 37°C for 24 hours. A loopful from incubated Tryptone soya broth was streaked onto the following media: Tryptic soya agar; MacConkey's agar plates; Aeromonas base agar; Rimler- Shotts agar (R.S.); Thiosulphate –Citrate –Bile –Sucrose (T.C.B.S) agar ; Eosin methylene blue agar (EMB); , blood agar plus 10 mcg /liter ampicillin, starch agar and milk agar media. All plates were incubated for 24hours at 37ºC.The developed colonies were picked up and subculture for purification. The purified colonies were morphologically identified by Gram stain and biochemical tests (Nicky, 2004; Guadalupe *et al*., 2009; Jayavignesh *et al*., 2011and Markey *et al*., 2013).

2.3.3. Genotypic detection of some virulence genes in Aeromonas species using polymerase chain reaction (PCR)

Genotyping detection of haemolysin (*hly*); *A. hydrophila* cytolytic enterotoxin (*Ahcytoen*); aerolysin (*aero*); cytotoxic enterotoxin (*act*) and cytotonic enterotoxins-heat-stable (*ast*) genes using conventional PCR in 16 random isolated Aeromonas spp. (10 *A. hydrophila* and 6 *A. caviae*), following QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR mastermix (Takara) with Code No. RR310Aand 1, 5% agarose gel electrophoreses (Sambrook *et al.,* 1989) using the Primers sequences, target genes, amplicons sizes and cycling conditions showed in Table (1).

3. RESULTS

The clinical examination of studied fish showed irregular hemorrhages all over the fish body especially at the ventral part of abdomen, base of the fins, and around the anal opening. Some fish showed congestion in the fins and its bases some had eroded fins, loss of fin membrane and sometimes loss of fin rays with grey patches at the tip of them(fins rot). Others showed eye cloudiness, detachment of scales and skin ulceration and abdominal distention. Internally these fishes showed abdominal dropsy with reddish ascetic exudates, liver paleness and enlargement in some fishes and congested with necrotic patches in other fishes; congested kidneys; congested and enlarged spleen and hemorrhagic enteritis that sometimes filled with yellow mucous like materials in some fishes.

The recovered isolates in this study are Gram –negative, straight rods with round end, non-capsulated, non-sporulated. Moreover, they grow well and showed white colonies on Tryptone soya agar, pale colonies then become pink on MacConkey's agar media. While on Rimler- Shotts medium (R.S) produced yellow convex colonies, on Aeromonas agar they give green colonies darker in center than emerging, only *A. hydrophila* strains give violet to metallic green sheen colonies on EMB media due to

lactose utilization; they give yellow colonies on Thiosulphate –citrate –bile –sucrose (T.C.B.S) agar due to fermentation of sucrose; showed large grayish circular, smooth, glistening colonies and surround by beta haemolysis and newly isolated strain have a pungent foul odor on blood agar; they hydrolysis starch on starch agar and detected by logus iodine due to amylase enzyme and showed a clear zone on milk agar media due to proteolysis of milk casein.

The results of bacteriological examination of examined fishes; in- vitro for the isolated strains and polymerase chain reaction (PCR) were tabulated in Tables (2&3) and Figures (1-5). According to biochemical reaction in table(4) The results revealed that, 125 Aeromonas species were isolated from the examined samples where *A. hydrophila* and *A. caviae* were identified. Accurately 114 (91.2 %) *A. hydrophila* strains, 63 (50.4%) and 51 (40.8%) were isolated from *C. gariepinus* and *O. niloticus* fishes respectively. Meanwhile, 11(8.8 %) *A. caviae* strains, 7 (5.6%) and 4 (3.2%) from *C. gariepinus* and *O. niloticus* fishes respectively.further PCR results for virulence genes in isolated Aeromonas strains indicated that, *aero* gene was detected in 9 out of 10 *A .hydrophila* studied strains and in 3 out of 6 *A. caviae* ; *hly* gene in 7 out of 10 *A .hydrophila* and in 2 out of 6 *A. caviae*; *Ahcytoen* gene in 6 out of 10 *A .hydrophila* and in 1 out of 6 *A. caviae* ; *act* gene in 6 out of 10 *A .hydrophila* and in 3 out of 6 *A. caviae* and *ast* gene in 7 out of 10 *A .hydrophila* and in 3 out of 6 *A. caviae* studied strains. Finally, the production of a wide array of virulence factors by isolated strains is indicative of their potential to cause diseases in fishes and humans.

Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	References
				Secondary denaturation	Annealing	Extension		
Haemolysin (*hly*)	CTATGAAAAAACTAAAAATAACTG CAGTATAAGTGGGGAAATGGAAAG	1500	94˚C 5 min.	94˚C 30 sec.	55˚C 1 min.	72˚C 1.5 min.	72˚C 12 min.	Yousr *et al*., 2007
A. hydrophila cytolytic enterotoxin *(Ahcytoen)*	GAGAAGGTGACCACCAAGAACAA AACTGACATCGGCCTTGAACTC	232	94˚C 5 min.	94˚C 30 sec.	56˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Cagatay and Şen, 2014
Aerolysin *(Aero)*	CACAGCCAATATGTCGGTGAAG GTCACCTTCTCGCTCAGGC	326	94˚C 5 min.	94˚C 30 sec.	52˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Singh *et al*., 2008
Cytotoxic enterotoxin *(act)*	AGAAGGTGACCACCACCAAGAACA AACTGACATCGGCCTTGAACTC	232	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Nawaz *et al*., 2010
Cytotonic enterotoxins - heat-stable (*ast*)	TCTCCATGCTTCCCTTCCACT GTGTAGGGATTGAAGAAGCCG	331	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	

Table (2): Prevalence of positive samples for Aeromonas species isolation among examined fishes

Fish type	No. of examined fish	No. of examined lesion samples	No. of Positive samples	Positive percentage	
				%*	%**
Nile tilapia (*o. niloticus*)	125	240	55	44.0	22.9
Catfish (*c. gariepinus*)	100	192	70	70.0	36.4
Total	225	432	125	55.6	28.9

*Percentage in relation to number of examined fish type

**Percentage in relation to no. of lesion samples in each raw

Table (3): Prevalence of Aeromonas species isolated from examined fishes

Fish type	No. of examined lesion samples	positive samples for Aeromonas species					
		A. hydrophila		*A. caviae*		Total	
		No.	%*	No.	%*	No.	%*
Nile tilapia (*O. niloticus*)	240	51	40.8	4	3.2	55	44.0
Cat fish (*C. gariepinus*)	192	63	50.4	7	5.6	70	56.0
Total	432	114	91.2	11	8.8	125	100.0

*Percentage in relation to number of Aeromonas species isolated (125)

Table (4): Biochemical reaction of Aeromonas species

Biochemical tests	*A. hydrophila*	*A.caviae*
Indole test		
	+ve	+ve
Methyl red	-ve	+ *ve*
Voges-Proskauer	+ve	- ve
Citrate utilization	+ve	+ve
H_2s production	+ve	- ve
Triple Sugar Iron slope	Yellow +g	yellow
β haemolysis	+ve	+ve
sugar fermentation		
Glucose	Ag	A
Fermentation of salicin	+ve	+ve
Mannitol	+ ve	+ve
Sucrose	+/-ve	-ve
Lactose		
	- ve	-ve
Urease test	- ve	-ve
Lysine decarboxylase		
	+ve+H2S	+ve
Oxidase test		
	+ve	+ve

+ve =positive - ve = Negative +/-ve =Most of isolated gave positive results Ag =Acid and gases

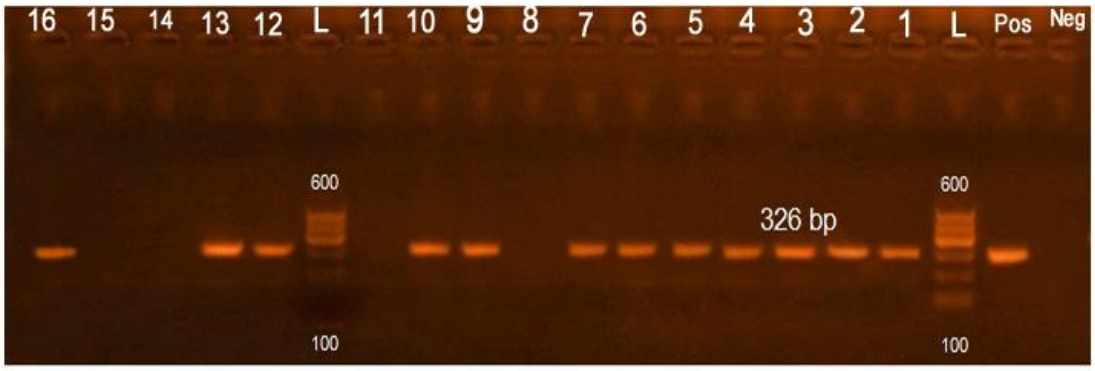

Fig. (1): PCR amplification of Aerolysin (*aero*) gene *0f a.hydrophila* and *a.caviae*

Lane L: 100-600 bp. DNA Ladder.

Neg.: Negative control. Pos.: Positive control (at 326 bp.).

Lane 1- 7,9&10: *A .hydrophila* (Positive). Lane 8: *A .hydrophila* (Negative).

Lane 12, 13 &16: *A. caviae* (Positive). Lane 11, 14 &15: *A. caviae* (Negative)

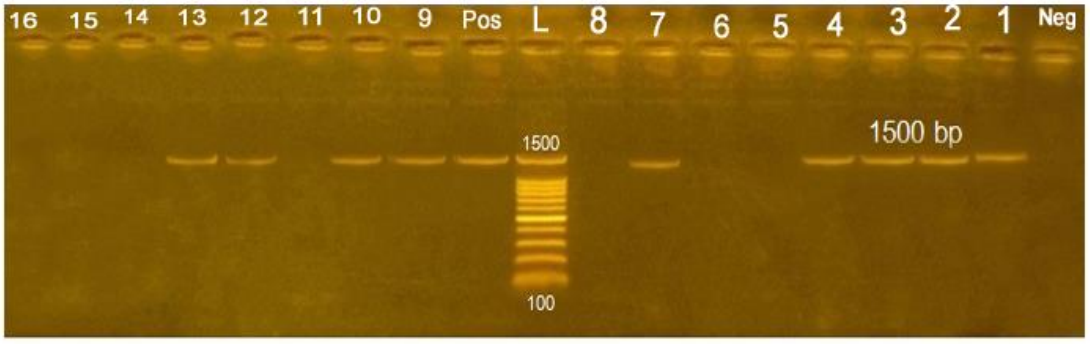

Fig. (2): PCR amplification of Haemolysin (*hly*) gene*0f a.hydrophila* and *a.caviae***.**

Lane L: 100-1500 bp. DNA Ladder.

Neg.: Negative control. Pos.: Positive control (at 1500 bp.).

Lane 1- 4, 7, 9 &10: *A .hydrophila* (Positive). Lane 5, 6 &8: *A .hydrophila* (Negative).

Lane 12&13: *A. caviae* (Positive). Lane 11, 14, 15 &16: *A. caviae* (Negative)

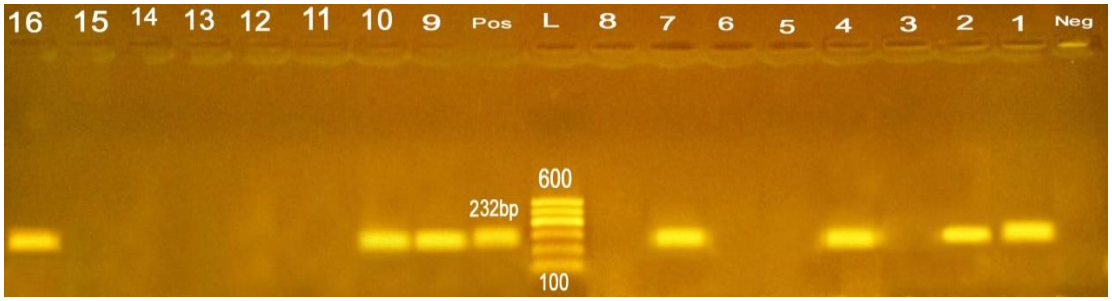

Fig. (3) PCR amplification of *A. hydrophila* cytolytic enterotoxin (*Ahcytoen*) gene of *A.hydrophila* and *A.caviae*.

Lane L: 100-600 bp. DNA Ladder.

Neg.: Negative control. Pos.: Positive control (at 232 bp.).

Lane 1, 2, 4, 7, 9&10: *A .hydrophila* (Positive). Lane 3, 5, 6&8: *A .hydrophila* (Negative).

Lane 16: *A. caviae* (Positive). Lane 11, 12, 13, 14&15: *A. caviae* (Negative)

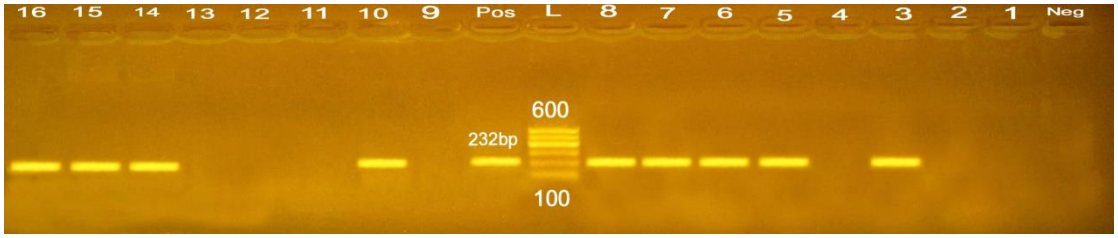

Fig. (4) PCR amplification of Cytotoxic enterotoxin (*act*) gene. of *A.hydrophila* and *A.caviae*.

Lane L: 100-600 bp. DNA Ladder.

Neg.: Negative control. Pos.: Positive control (at 232 bp.).

Lane 3, 5-8 &10: *A .hydrophila* (Positive). Lane 1, 2, 4&9: *A .hydrophila* (Negative).

Lane 14, 15&16: *A. caviae* (Positive). Lane 11, 12&13: *A. caviae* (Negative)

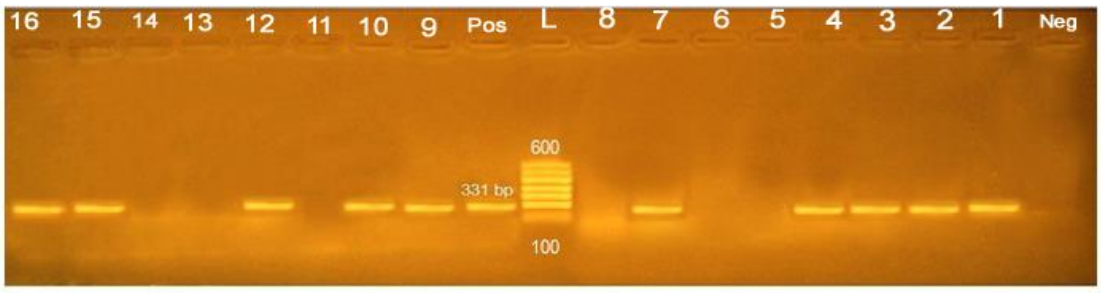

Fig. (5): PCR amplification of Cytotonic enterotoxins- heat-stable (*ast*) gene. of *A.hydrophila* and *A.caviae*. Lane L: 100-600 bp. DNA Ladder

Neg.: Negative control. Pos.: Positive control (at 331 bp.).

Lane 1- 4, 7, 9&10: *A .hydrophila* (Positive). Lane 5, 6&8: *A .hydrophila* (Negative).

Lane 12, 15&16: *A. caviae* (Positive). Lane 11, 13&14: *A. caviae* (Negative)

4. DISCUSSION

The present study was planned for determination the prevalence of Aeromonas infection in fresh water fishes, Nile tilapia fish (*O. niloticus*) and. Cat fish (*C. gariepinus*) and phenotypic characterization of Aeromonas species and detection of some virulence genes in some isolated strains.

The results of clinical and postmortem examinations of studied fish were similar to that reported by Noor El- Deen *et al.* (2014);Ibrahim- Lamis (2015); Paul *et al.* (2015) and Sayed(2017). The prevalence of Aeromonas septicemia with Aeromonas species isolation (Table, 2) revealed that, 125 out of 225 examined fish (55.6%) and of 432 lesion samples (28.9 %): represented as 55 positive samples (44.0% and 22.9%) from 125 *O. niloticus* examined fish and 240 lesion samples; meanwhile, 70 (70.0% and 36.4%) from 100 *C. gariepinus* examined fish and 192 lesion samples were positive for Aeromonas species isolation. These results came in accordance with these obtained by Yucel *et al.* (2005) and El- Dien *et al.* (2010) and disagreed with Ibrahim- Lamis (2015) who recorded higher incidence.

The results of bacteriological examination (Table, 3) revealed that, 125 Aeromonas species were isolated from examined samples where *A. hydrophila* and *A. caviae* were the only species isolated. Similar results were recorded by Stratev *et al*. (2012). A total of 114 (91.2 %) *A. hydrophila* strains, 63 (50.4%) and 51 (40.8%) were isolated from *C. garicpinus* and *O. niloticus* fishes respectively. Meanwhile, 11(8.8 %) *A. caviae* strains, 7 (5.6%) and 4 (3.2%) from *C. lazera* and *O. niloticus* fishes respectively. These results agree with those of Abu- Leila (2005); Ibrahim- Lamis (2015) and Sayed(2017). Meanwhile lower incidence was recorded byNawaz *et al.* (2006); and Noor El- Deen *et al.* (2014) The recoded results are in concordance with Hayes (2000) who concluded that outbreaks of *A. hydrophila* and *A. caviae* were usually associated with change in environmental conditions. Stressors including overcrowding, high temperature, sudden change in temperature, poor nutritional state, and fungal or parasitic infection that made stress on fish and increase its susceptibility to the infection. In addition, fish contaminated with *A. hydrophila* and *A. caviae* could be hazardous, especially for sensitive populations, such as children, elderly persons and immunocompromised people (Herrera *et al*., 2006 and Stratev *et al.*, 2016). The morphological characteristics of the colonies, Gram staining and the biochemical profile of Aeromonas species isolated such as the fermentation of certain sugars or enzymatic reaction as protease and lipase was similar to those previously reported (Songer and Post, 2005; Jayavignesh *et al.*,2011 and Kareem - Niamah ,2012) .

Some strains of Aeromonas are reported to be invasive to epithelial cells and one of the major virulence factors in gastroenteritis is aerolysin (Chu and Lu, 2005), the results of PCR for amplification of *aero* gene in *A .hydrophila and A. caviae* strains (Fig., 1) showed that, the *aero* gene was amplified in 9 out of 10 *A .hydrophila* studied strains and in 3 out of 6 *A. caviae* studied strains giving product of 326 bp. Similar results were decided by Nam and Joh (2007) ;Yousr *et al.* (2007); Yogananth *et al*.(2009); Kareem -Niamah (2012); Oliveira-Samira *et al.* (2012) ; Ye *et al*. (2013) ;Aravena *et al*. (2014) ; Furmanek (2014) ; Stratev *et al.* (2016) and Sayed 2017). Meanwhile, they were disagreed with that recorded by Gonzalez-Serrano *et al*.(2002),

Kore *et al*. (2014) and Ibrahim- Lamis (2015) who failed to detect *aero* virulent gene in these strains and with Aravena *et al.* (2014) in *A. caviae* strains.

In addition, the results of PCR for amplification of *hly* gene in *A .hydrophila and A. caviae* strains (Fig., 2) showed that, the *hly* gene was amplified in 7 out of 10 *A .hydrophila* studied strains and in 2 out of 6 *A. caviae* studied strains giving product of 1500 bp. Similar results were decided by Wang *et al*. (2003); Nam and Joh (2007);Yousr *et al.* (2007); Yogananth *et al*. (2009) ; Cagatay and Şen (2014) and Stratev *et al.* (2016). Regarding to the results of PCR for amplification of *Ahcytoen* gene in *A .hydrophila and A. caviae* strains (Fig., 3) showed that, the *Ahcytoen* gene was amplified in 6 out of 10 *A .hydrophila* studied strains and in 1 out of 6 *A. caviae* studied strains giving product of 332 bp. Similar results were recorded by Sechi *et al*. (2002); Wang *et al*. (2003); Sechi *et al.* (2004) ; Sarkar *et al*. (2013) and Cagatay and Şen (2014). For *act* gene, it was amplified in 6 out of 10 *A .hydrophila* studied strains and in 3 out of 6 *A. caviae* studied strains giving product of 332 bp. as shown in Fig. (4). These results agreed with those of Abdullah *et al.* (2003); Ashok *et al.* (2009); Bin Kingombe *et al*.(2010); Nawaz *et al*. (2010); Ye *et al*. (2013) ;Furmanek (2014) and Sayed(2017). Moreover, the *ast* gene was amplified in 7 out of 10 *A .hydrophila* studied strains and in 3 out of 6 *A. caviae* studied strains giving product of 331 bp. as shown in Fig. (5). Similar findings were recorded by Sha *et al.* (2002); Ashok *et al.* (2009); Bin Kingombe *et al*.(2010); Ye *et al.* (2013) and Aravena *et al.* (2014).

Finally, from results of the present work it could be concluded that, Aeromonas species specially, *A. hydrophila* and *A. caviae* are important pathogens causes septicemia in fish. Moreover, most isolated strains were enterotoxigenic ones, as they had haemolytic; amylase; proteolytic and lipolytic activities. In addition, the production of a wide array of virulence factors by them is indicative of their potential to cause diseases in fishes and humans.

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