Nature and Science

Websites:
http://www.sciencepub.net/nature
http://www.sciencepub.net

Emails:
naturesciencej@gmail.com
editor@sciencepub.net

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Phenotypic and molecular detection of Aeromonas and pseudomonas species isolated from fish with special reference to their virulence factors

Ashraf, A. Abd El- Tawab[1], Ahmed, A.A. Maarouf[2], Fatma, I. El Hofy[1], Amany, O. Salim[2] and Aya, A.M. El-sayed[3]

Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Benha University, Egypt.
2Animal Health Research "Benha branch". Agriculture Research Center, Egypt.
3Veterinarian.
mohamedebrahim30901988@gmail.com

Abstract: The present study was conducted on 250 diseased Nile tilapia (*Oreochromis niloticus*) fish of various sizes were collected from different fish markets at Kaliobia Governorate, Egypt, during the period from May (2017) to January (2019) for inspection of Aeromonas and Pseudomonas strains. Samples were collected from apparently pathognomic lesions in muscle, liver, kidney, intestine and spleen for bacteriological examination. The results revealed that, 161 Aeromonas and Pseudomonas species; 118 Aeromonas (73.3%) and 43 Pseudomonas species (26.7%) were isolated mostly from 50 muscle lesion samples followed by 41 liver samples then 35 kidney samples; 32 intestine samples and 3 spleen lesion samples. Most isolated Aeromonas and Pseudomonas strains were enterotoxigenic ones, as they had haemolytic; amylase activity; proteolytic; lipolytic; Lecithinase activities and had the ability for biofilm production. PCR results showed that, specific gene of *A. hydrophila* **(16SrRNA)** and polar flagella gene (***fla***) genes were detected in all 8 studied strains; aerolysin (***aer*o**); *A. hydrophila* cytolytic enterotoxin (**AHcytoen**); cytotonic enterotoxins- heat-stable (***ast***) virulence genes were detected in 5; 3 and 5 out of 8 *A. hydrophila* studied strains. In addition, specific gene of *Ps. aeruginosa* **(16SrDNA)**; outer membrane lipoprotein L (***opr*L**) and flagellin C (***fli*C**) genes were detected in all 8 studied *Ps. aeruginosa* strains; exotoxin A (***tox*A**) and exotoxin S (***exo*S**) virulence genes were detected in 5 and 6 out of 8 studied ones.
[Ashraf, A. Abd El-Tawab, Ahmed, A.A. Maarouf, Fatma, I. El Hofy, Amany, O. Salim and Aya, A.M. El-sayed. **Phenotypic and molecular detection of Aeromonas and pseudomonas species isolated from fish with special reference to their virulence factors.** *Nat Sci* 2019;17(12):194-205]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 27. doi:10.7537/marsnsj171219.27.

Key words: Fish, Aeromonas, Pseudomonas, Lecithinase, virulence genes

1. Introduction

Among the etiological agents of bacterial fish diseases, Aeromonas and Pseudomonas are considered as the most important fish pathogens which are responsible for ulcer type diseases including ulcerative syndrome leading to high mortalities and high economic losses (**Zilberberg and Shorr, 2009; Shayo *et al*., 2012** and **Hanna *et al*., 2014**). The disease is characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascites and exophthalmia (**Eissa *et al*., 2010** and **Hanna *et al*., 2014**). Members of the Aeromonas and Pseudomonas, are Gram-negative rods, either straight or curved facultative anaerobes, catalase-positive and most are motile by polar flagella. Their nutritional requirements are very simple and most will grow on common laboratory media. They are widespread in freshwater, sewage, soil and their numbers rise with the amount of organic matter present **(Markey *et al.*, 2013**). Aeromonads produce extracellular enzymes (haemolysins, lipases, proteases, β-lactamases, amylases, chitinases and nucleases) involved in their ecology, survival and pathogenicity (**Stratev *et al*., 2015**). In addition, the pathogenicity of *A. hydrophila* strains 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).** Exotoxins are major virulence factors of Aeromonads that include a cytotoxic heat-labile enterotoxin (***act***), also known as aerolysin/hemolysin; a cytotonic heat-labile enterotoxin (***alt***), known as lipase, extracellular lipase, or phospholipase and (***ast***) a cytotonic heat- stable enterotoxin (**Bin Kingombe *et al*., 2010**). Moreover, *Ps. aeruginosa* and *Ps. fluorescens* are considered problematic pathogens as they possesses cell-associated virulence factors such as (pili, flagella, lipopolysaccharide, and alginate/biofilm), they also produces a number of extracellular products such as

protein exotoxin A, proteases, type III secretion system exoenzymes, rhamnolipid, haemolysin with lecithinase activity; elastase (***las* B** and ***las* A**), siderophores (pyochelin, pyocyanin, and pyoverdine by *Ps. aeruginosa* and thioquinolobactin by *Ps. fluorescens*) and phospholipase C. (**Mavrodi *et al.*, 2001 and Markey *et al.*, 2013)**. These virulence factors all play a role in disease pathogenesis. As Aeromonas and Pseudomonas are considered one of the most important fish pathogens and can be a problem for human consumers too and fish had attained a great economic importance in Egypt, so, the present study was conducted to throw light over their infection in fresh water fish, Nile tilapia fish (*O. Niloticus*), beside phenotypic; genotypic characterization and detection of some virulence factors in some isolated *A. hydrophila* and *Ps. aeruginosa* strains by using P C R.

2. Material and Methods

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

Target M.O.	Target gene		Primer sequence (5'-3')	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	References
						Secondary	Annealing	Extension		
A. hydrophila	*A. hydrophila 16S rRNA*	F	GAAAGGTTGATGCCTAATACGTA	625 bp.	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 45 sec.	72°C 10 min.	Gordon *et al.*(2007)
		R	CGTGCTGGCAACAAAGGACAG							
	Aero	F	CACAGCCAATATGTCGGTGAAG	326 bp.	94°C 5 min.	94°C 30 sec.	52°C 40 sec.	72°C 40 sec.	72°C 10 min.	Singh et al. (2008)
		R	GTCACCTTCTCGCTCAGGC							
	AHcytoen	F	GAGAAGGTGACCACCAAGAACAA	232 bp.	94°C 5 min.	94°C 30 sec.	56°C 30 sec.	72°C 30 sec.	72°C 7 min.	Cagatay and Şen(2014)
		R	AACTGACATCGGCCTTGAACTC							
	ast	F	TCTCCATGCTTCCCTTCCACT	331 bp.	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72°C 7min.	Nawaz *et al.* (2010)
		R	GTGTAGGGATTGAAGAAGCCG							
	fla	F	TCCAACCGTYTGACCTC	608 bp.	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	Nawaz *et al.* (2010)
		R	GMYTGGTTGCGRATGGT							
Ps. aeruginosa	*Ps. aeruginosa 16S rDNA*	F	GGGGGATCTTCGGACCTCA	956 bp.	94°C 5 min.	94°C 30 sec.	52°C 45 sec	72°C 1 min.	72°C 10 min.	Spilker *et al.* (2004)
		R	TCCTTAGAGTGCCCACCCG							
	oprL	F	ATG GAA ATG CTG AAA TTC GGC	504 bp.	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	Xu *et al.*(2004)
		R	CTT CTT CAG CTC GAC GCG ACG							
	toxA	F	GACAACGCCCTCAGCATCACCAGC	396 bp.	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	Matar *et al.* (2002)
		R	CGCTGGCCCATTCGCTCCAGCGCT							
	exoS	F	GCGAGGTCAGCAGAGTATCG	118 bp.	94°C 5 min.	94°C 30 sec.	55°C 30 sec.	72°C 30 sec.	72°C 7 min	Winstanley *et al.*(2005)
		R	TTCGGCGTCACTGTGGATGC							
	fliC	F	TGAACGTGGCTACCAAGAACG	180 bp.	94°C 5 min.	94°C 30 sec.	56.2°C 30 sec.	72°C 30 sec.	72°C 7 min	Ghadaksaz *et al.*(2015)
		R	TCTGCAGTTGCTTCACTTCGC							

The present study was conducted on 250 diseased Nile tilapia (*O. niloticus*) fish of various sizes were collected from different fish markets at Kaliobia Governorate, Egypt, during the period from May (2017) to January (2019) for inspection of Aeromonas and Pseudomonas strains. After clinical and postmortem examination 445 samples were collected from apparently pathognomic lesions in muscle, liver, kidney, intestine and spleen by a number of 139, 118, 99, 70 and 19 respectively.

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 Tryptone soya agar and MacConkey's agar plates and incubated for 24 hours at 37ºC. The pale colonies on MacConkey's agar and white or greenish colonies on Tryptone soya agar were picked up and the following tests (Oxidase test and Catalase test) were performed. The suspected colonies that gave (Oxidase +ve and Catalase +ve) were taken and cultivated on the following media: Aeromonas base agar; Rimler- Shotts agar (R.S.) and Eosin methylene blue agar (EMB); Pseudomonas agar and Pseudomonas Cetrimide agar then incubated for another 24-48 hours at25°C and 37ºC, suspected colonies were picked up and purified by further sub culturing on nutrient agar and kept in Semi-solid nutrient agar. The purified colonies were morphologically identified by Gram stain and biochemical tests (**Quinn *et al.*, 2002; Austin and Austin, 2007 and Markey *et al.*, 2013**).

The In-Vitro sensitivity test was done on each isolated Aeromonas and Pseudomonas species strain to study its anti-microbial sensitivity according to

(Koneman *et al.*, 1997).

The phenotypic detection of virulence factors for isolated Aeromonas and Pseudomonas species was done through studying the haemolytic activities following **Quinn *et al.* (2002);** amylase activity **(Palumbo *et al.* 1985)**; proteolytic activities (**Marcy and Pruett,2001);** lipolytic activities (**Haas,2001);** lecithinase activities (**Anguita *et al.*,1993)** and the biofilm formation of them using tube method**.** In addition to**,** genotyping detection for specific gene of *A. hydrophila* **(16SrRNA);** aerolysin (***aer*o**); *A. hydrophila* cytolytic enterotoxin (**AHcytoen**); cytotonic enterotoxins- heat-stable (***ast***) and polar flagella gene (***fla***) in 8 random *A. hydrophila* strains, beside, specific gene of *Ps. aeruginosa* **(16SrDNA)**; outer membrane lipoprotein L (***opr*L**); exotoxin S gene (***exo*S**); exotoxin A gene (***tox*A**) and flagellin C gene (***fli*C**) in 8 random isolated *Ps. aeruginosa* strains using conventional PCR, 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 results of bacteriological examination of examined fish; in- vitro sensitivity tests; phenotypic detection of virulence factors for the isolated strains and polymerase chain reaction (PCR) were tabulated in Tables (2 & 3) and Figures (1-10).

Table (2): Prevalence and distribution of Aeromonas and Pseudomonas species isolated from examined lesion samples

Isolates species	Lesion samples										Total	
	Muscle		Liver		Kidney		Intestine		Spleen			
	No.	%*	No.	%*	No.	%*	No.	%*	No.	%*	No.	%*
Aeromonas species	36	22.4	29	18.0	26	16.1	24	14.9	3	1.9	118	73.3
A. hydrophila	29	18.0	25	15.5	23	14.3	22	13.7	3	1.9	102	63.4
A. caviae	7	4.4	4	2.5	3	1.9	2	1.2	0	0.0	16	9.9
Pseudomonas species	14	8.7	12	7.5	9	5.6	8	5.0	0	0.0	43	26.7
Ps. aeruginosa	11	6.8	7	4.4	5	3.1	6	3.7	0	0.0	29	18.0
Ps. fluorescens	3	1.9	5	3.1	4	2.5	2	1.2	0	0.0	14	8.7
Total	50	31.0	41	25.5	35	21.7	32	19.9	3	1.9	161	100

%* percentage in relation to total number of isolated species (161)

Table (3): Phenotypic virulence factors of Aeromonas and Pseudomonas isolates

Phenotypic virulence activities	Aeromonas strains				Pseudomonas strains			
	A. hydrophila (102)		*A. caviae* (16)		*Ps. Aeruginosa* (29)		*Ps. Fluorescens* (14)	
	No.	%*	No.	%*	No.	%*	No.	%*
Haemolytic activity	93	91.2	13	81.3	26	89.7	9	64.3
Starchhydrolysis (amy lase activity)	98	96.1	14	87.5	27	93.1	11	78.6
Proteolytic (caseinase) activity	95	93.1	12	75.0	27	93.1	10	71.4
Lipolytic activity	90	88.2	10	62.5	25	86.2	10	71.4
Lecithinase activity	89	87.3	9	56.3	24	82.8	9	64.3
Biofilm production	91	89.2	11	68.8	27	93.1	11	78.6

*Percentage in relation to number of each Aeromonas and Pseudomonas species isolated

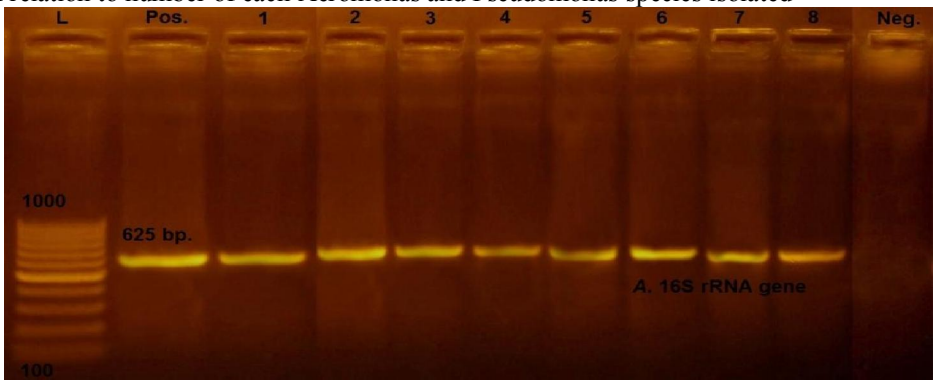

Fig. (1): Agarose Gel electrophoresis of specific gene of *A.hydrophila* (16SrRNA)
Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control (at 625 bp.)
Lane 1-8: *A.hydrophila* (Positive)

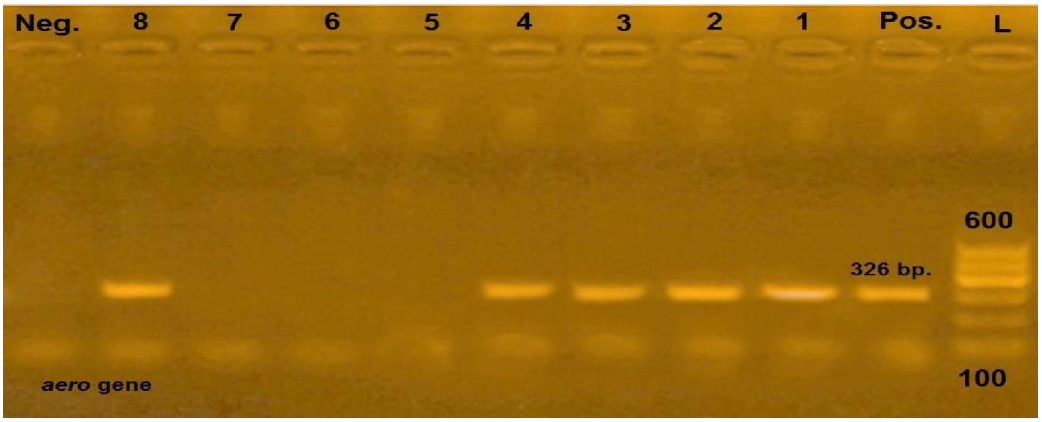

Fig. (2): PCR amplification of Aerolysin (*aero*) gene on agarose gel 1.5%.
Lane L: 100-600 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control (at 326 bp.)
Lane 1- 4 & 8: *A.hydrophila* (Positive) Lane 5, 6 & 7: *A.hydrophila* (Negative)

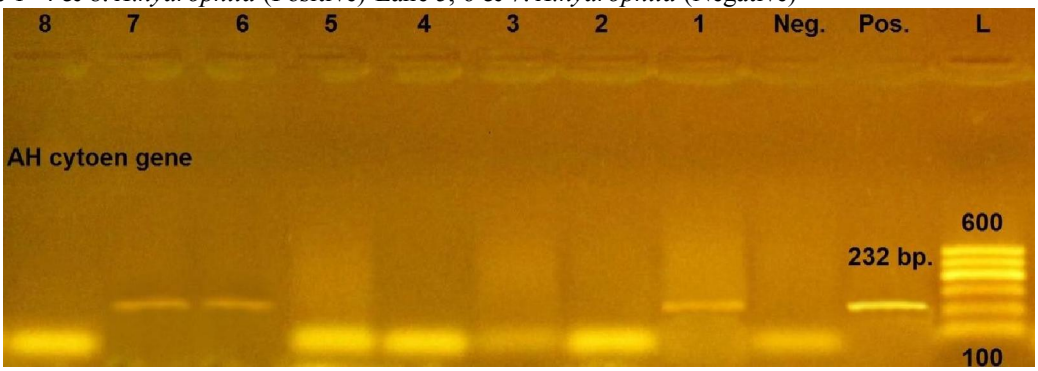

Fig. (3): PCR amplification of *A. hydrophila* cytolytic enterotoxin (*AHcytoen*) on agarose gel 1.5%.
Lane L: 100-600 bp. DNA Ladder.
Neg.: Negative control. Pos.: Positive control (at 232 bp.).
Lane 1, 6, & 7: *A.hydrophila* (Positive). Lane 2,3,4, 5 & 8: *A.hydrophila* (Negative)

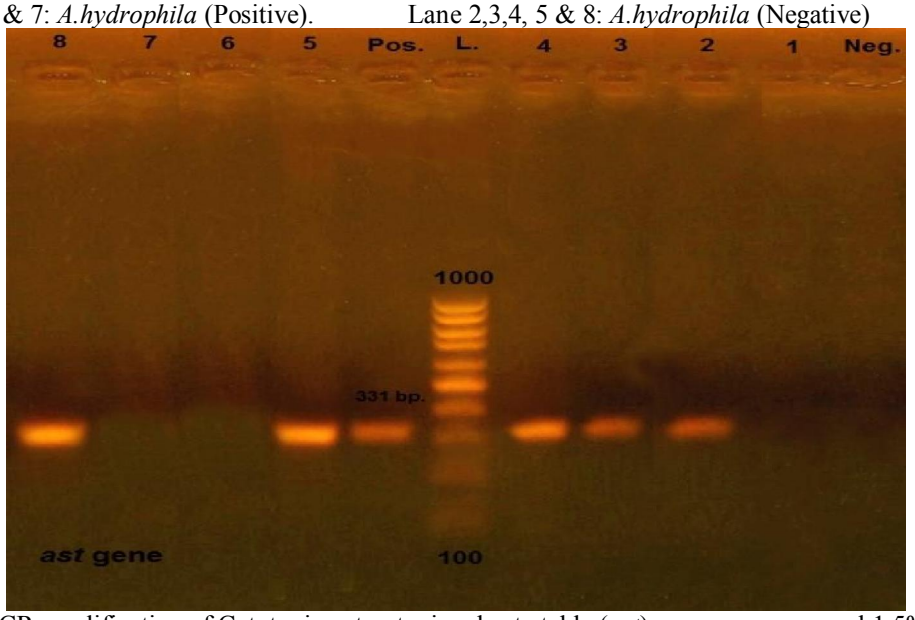

Fig. (4): PCR amplification of Cytotonic enterotoxins- heat-stable (*ast*) gene on agarose gel 1.5%.
Lane L: 100-1000 bp. DNA Ladder.
Neg.: Negative control. Pos.: Positive control (at 331 bp.).
Lane 2, 3, 4, 5 & 8: *A.hydrophila* (Positive). Lane 1, 6 & 7: *A.hydrophila* (Negative).

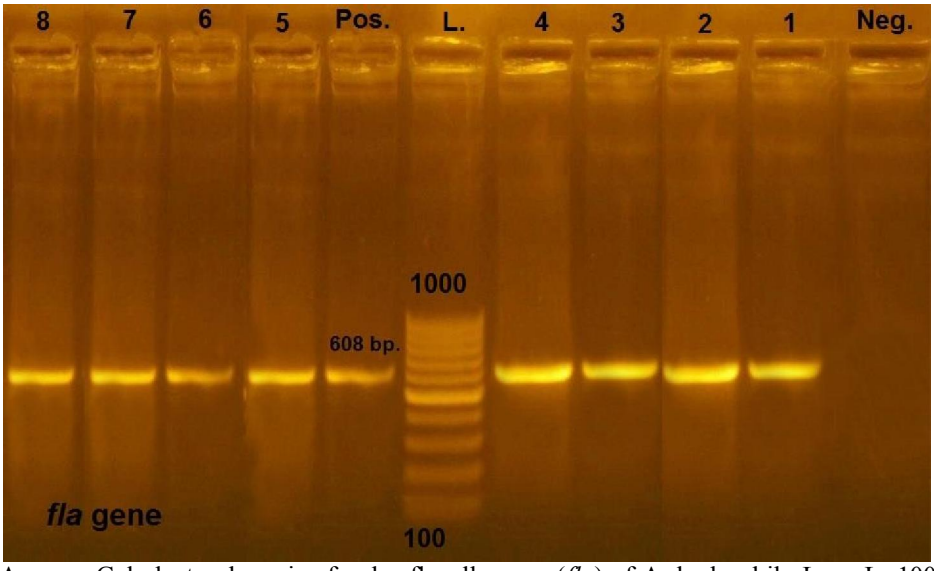

Fig. (5): Agarose Gel electrophoresis of polar flagella gene (*fla*) of A. hydrophila Lane L: 100-1000 bp DNA Ladder, Neg.: Negative controPos.: Positive control (at 608 bp) Lane 1 – 8: *A. hydrophila* (Positive)

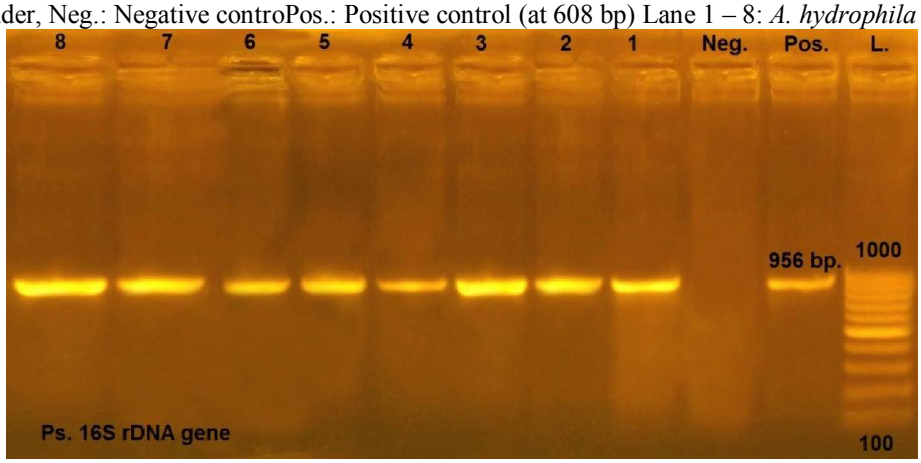

Fig. (6): Agarose Gel electrophoresis of specific gene of Ps. aeruginosa (16SrDNA) Lane L: 100-1000 bp DNA Ladder. Neg.: Negative control. Pos.: Positive control (at 956bp.) Lane 1- 8: Ps. aeruginosa (Positive)

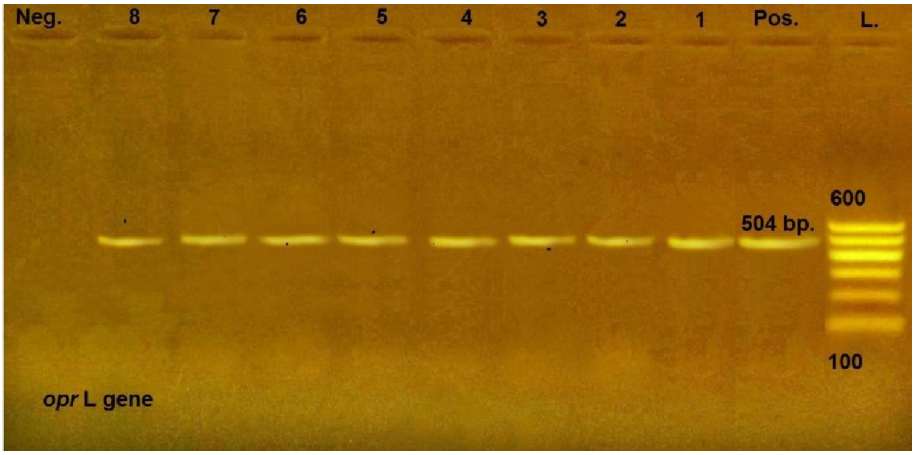

Fig. (7): Agarose Gel electrophoresis of outer membrane lipoprotein L gene (*opr*L) of *Ps. aeruginosa* Lane L: 100-600 bp DNA Ladder. Neg.: Negative control.
Pos.: Positive control (at 504 bp) Lane 1- 8: *Ps. aeruginosa* (Positive)

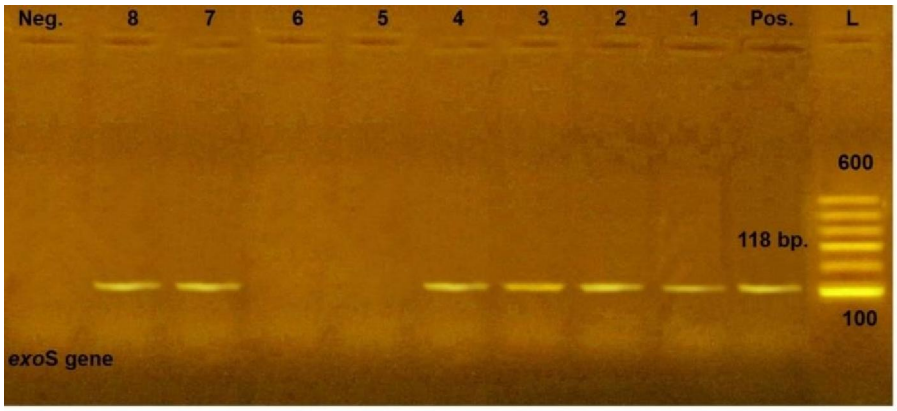

Fig. (8): Agarose Gel electrophoresis of exotoxin S (exoS) gene of *Ps. aeruginosa*
Lane L: 100-600 bp DNA Ladder. Neg.: Negative control Pos.: Positive control (at 118 bp)
Lane 1-4,7 & 8: *Ps. aeruginosa* (Positive) Lane 5 & 6: *Ps. aeruginosa* (Negative)

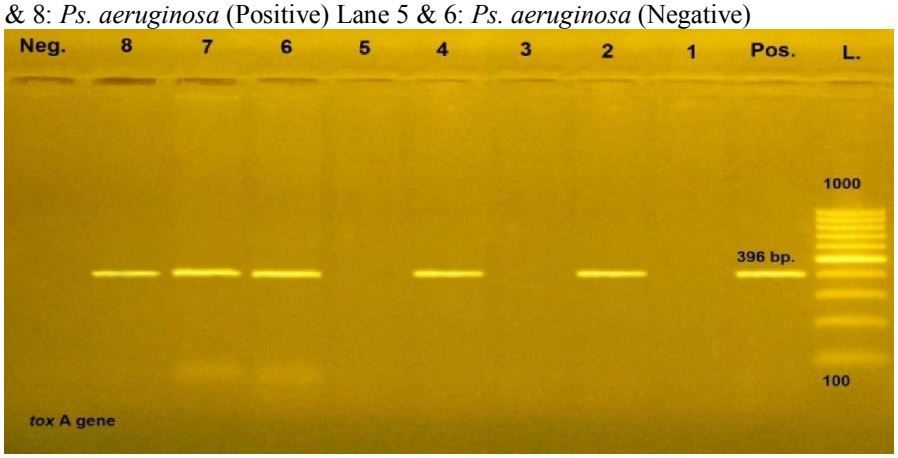

Fig. (9): Agarose Gel electrophoresis of exotoxin S (*tox*A) gene of *Ps. aeruginosa*
Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control Pos.: Positive control (at 396 bp.)
Lane 2, 4, 6, 7 & 8: *Ps. aeruginosa* (Positive) Lane 1, 3 & 5: *Ps. aeruginosa* (Negative)

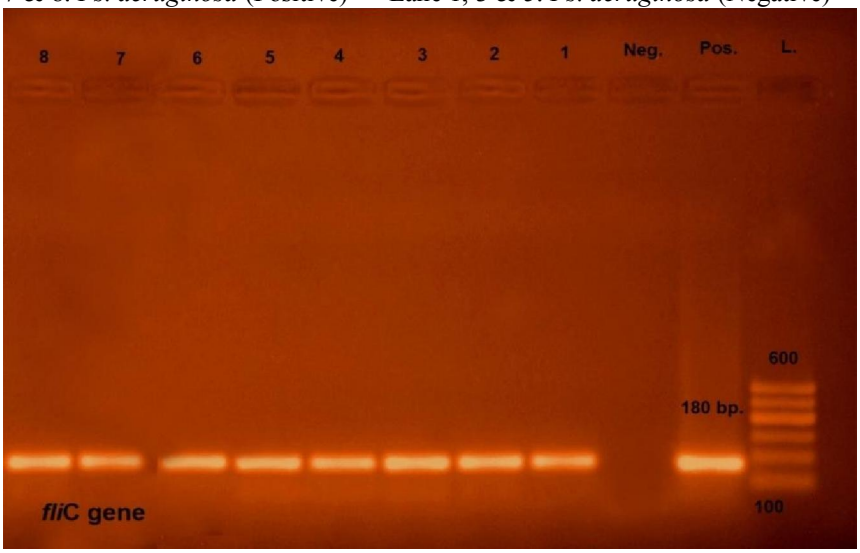

Fig. (10): Agarose Gel electrophoresis of flagellin C gene (*fli*C) gene of *Ps. aeruginosa*
Lane L: 100-600 bp DNA Ladder. Neg.: Negative control.
Pos.: Positive control (at 180 bp.) Lane 1- 8: *Ps. aeruginosa* (Positive)

4. Discussion

Aeromonas and Pseudomonas species are widely distributed microorganisms that responsible for wide range spectrum of ulcer type diseases among fish leading to high mortalities, high economic losses and decreases fish farm efficiencies (**Roberts, 2001** and **Shayo *et al*., 2012),** beside their role in gastrointestinal and extra intestinal infections in humans (**Zilberberg and Shorr, 2009** and **Parker and Shaw, 2011).**

. The results recorded in this study revealed that, 128 out of 250 examined fish (51.2%) were positive for Aeromonas and Pseudomonas species isolation, where 95(74.2%) were positive with pure single cultures; 85 Aeromonas spp. (66.4%) and 10 Pseudomonas spp. (7.8%) as well as 33(25.8%) mixed ones. These results came in accordance with that obtained by **El- Dien *et al.* (2010)**; **Shayo *et al.* (2012); Abd El Tawab *et al.* (2016)** and **Abd El Tawab *et al.* (2017).** The results of bacteriological examination (Table, 2) revealed that, 161 Aeromonas and Pseudomonas species; 118 Aeromonas (73.3%) and 43 Pseudomonas species (26.7%) were isolated mostly from 50 muscle lesion samples (31.0%) followed by 41 liver samples (25.5%) then 35 kidney samples.

(21.7%); 32 intestine samples (19.9%) and 3 spleen lesion samples (1.9%). Nearly similar results were recorded by **El-Hady and Samy (2011); Hanna *et al*. (2014)**; **Ibrahim- Lamis (2015) and Maarouf et *al*. (2017)**. Moreover, 36(22.4%) Aeromonas species were isolated from muscle lesion samples (29 *A. hydrophila*, 18.0% and 7 *A. caviae*, 4.4%); 29 (18.0%) from liver (25 A. *hydrophila*, 15.5% and 4 *A. caviae*, 2.5%); 26 (16.1%) from kidney (23 A. *hydrophila*, 14.3% and 3 *A. caviae*, 1.9%); 24 (14.9%) from intestine (22 *A. hydrophila*, 13.7% and 2 *A. caviae*, 1.2%) and 3 (1.9%) from spleen lesion samples that was *A. hydrophila* only. These results agree with those of **Thayumanavan *et al.* (2003)**; **Ibrahim- Lamis (2015)**; **Abd El Tawab *et al*. (2017) and Maarouf *et al.* (2017)**. In addition, 14(8.7%) Pseudomonas species were isolated from muscle lesion samples (11 *Ps. aeruginosa*, 6.8% and 3 *Ps. fluorescens*, 1.9%); 12 (7.5%) from liver (7 *Ps. aeruginosa*, 4.4% and 5 *Ps. fluorescens*, 3.1%); 9 (5.6%) from kidney (5 *Ps. aeruginosa*, 3.1% and 4 *Ps. fluorescens*, 2.5%) and 8 (5.0%) from intestine (6 *Ps. aeruginosa*, 3.7% and 2 *Ps. fluorescens*, 1.2%) but both Pseudomonas species were failed to be isolated from spleen samples. Nearly similar results were recorded by **Sakr and Abd El-Rhman (2008); Eissa *et al*., (2010); El-Hady and Samy (2011); Hanna *et al*., (2014) and Abd El Tawab *et al*. (2016).**

The results of phenotypic Virulence factors of isolated Aeromonas and Pseudomonas strains (Table, 3) showed that, 93 *A. hydrophila* (91.2%); 13 *A. caviae* (81.3%); 26 *Ps. aeruginosa* (89.7%) and 9 *Ps. fluorescens* (64.3%) isolated strains had haemolytic activity, as Aeromonas strains showed large grayish circular, smooth, glistening colonies and surround by beta haemolysis and newly isolated strain have a pungent foul odour on blood agar plus 10 ug /liter ampicillin. Similar results were recorded by **Singh *et al.* (2008); Kozinska and Pekala (2010)**; **Stratev *et al*. (2015) and Abd El Tawab *et al*. (2017).** Meanwhile, Pseudomonas strains showed irregular colonies surround by a clear zone of β-haemolysis with green-blue pyocyanin pigment is most obvious in areas of heaviest growth, *Ps. aeruginosa* on blood agar and *Ps. fluorescens* showed narrow zone of β-haemolysis. These results were agreed with those of **Jvo (2010)**; **Kamel *et al.* (2011)** and **Panda *et al.* (2013)**. For starch hydrolysis (amylase activity), 98 *A. hydrophila* (96.1%); 14 *A. caviae* (87.5%); 27 *Ps. aeruginosa* (93.1%) and 11 *Ps. fluorescens* (78.6%) isolated strains hydrolyzed starch on starch agar and detected by logus iodine due to amylase enzyme. Similar results for Aeromonas strains were recorded by **Pemberton *et al*. (1997); Sreedharan *et al*. (2012) and Abd El Tawab *et al.* (2017)** for Aeromonas strains and for Pseudomonas strains with **Tole *et al*. (2016); Alam and Imran (2018) and Mankar and Barate (2018).** For Proteolytic (caseinase) activity, 95 *A. hydrophila* (93.1%); 12 *A. caviae* (75.0 %); 27 *Ps. aeruginosa* (93.1%) and 10 *Ps. fluorescens* (71.4%) isolated strains had protease enzyme that was shown by the formation of a clear zone on milk agar media due to proteolysis of milk casein. Similar results for Aeromonas strains were obtained by **Castro-Escarpulli *et al*. (2003); Zhu-DaLing *et al.* (2006)**; **Sreedharan *et al.* (2012) and Stratev *et al.* (2015)** and for Pseudomonas strains with **Pollack (2000)**; **Jvo (2010)** and **Patil and Chaudhairy (2011)**. The lipolytic activity showed that, 90 *A. hydrophila* (88.2%); 10 *A. caviae* (62.5%); 25 *Ps. aeruginosa* (86.2%) and 10 *Ps. fluorescens* (71.4%) isolated strains presented lipolytic activity on agar supplemented with tributyrin and were detected by a transparent zone surrounding the colony on an opaque background. Similar results for Aeromonas strains were recorded by **Gonzalez-Serrano *et al.* (2002); Castro-Escarpulli *et al.* (2003); Sreedharan *et al.* (2012) and Stratev *et al.* (2015)** and with those recorded by **Picot *et al.* (2001); Jvo (2010)** and **Stoyanova *et al.* (2012)** for Pseudomonas strains**.** Also**,** 89 *A. hydrophila* (87.3%); 9 *A. caviae* (56.3%); 24 *Ps. aeruginosa* (82.8%) and 9 *Ps. fluorescens* (64.3%) isolated strains had lecithinase activity that was clearly marked by an opaque zone extending from the edge of the colony. These results were agreed with those of **Gonzalez-Serrano *et al.* (2002); Sreedharan *et al.* (2012)**; **Stratev *et al.* (2015) and Abd El**

Tawab *et al.* (2017) for Aeromonas strains and with those of **Pollack (2000) and Jvo (2010)** for Pseudomonas strains**.** Moreover, most isolated Aeromonas and Pseudomonas strains were multi-drug resistant where 91 *A. hydrophila* (89.2%); 11 *A. caviae* (68.8%); 27 *Ps. aeruginosa* (93.1%) and 11 *Ps. fluorescens* (78.6%) strains had the ability for biofilm production that was clearly marked by a visible film lined the wall and the bottom of the tube. These results came in harmony with those of **Castro-Escarpulli *et al*. (2003); Stratev *et al.* (2015)** and **Maarouf *et al*. (2017)** for Aeromonas strains and with those of **Pollack (2000); Meliani and Bensoltane (2015)**; **Banu *et al.* (2017); Omar *et al.* (2017)** and **Abdel-Haq- Fatma El- Zahraa (2018)** for Pseudomonas strains**.** So, most isolated Aeromonas and Pseudomonas strains were enterotoxigenic ones, as they had haemolytic; proteolytic; lipolytic; Lecithinase activities and had the ability for biofilm production.

The genotypic identification and detection of some virulence genes in isolated *A. hydrophila* and *Ps. aeruginosa* strains using PCR results showed that, specific gene of *A. hydrophila* (**16SrRNA)** and polar flagella gene (***fla***) genes were detected in all 8 studied strains; aerolysin (***aero***); *A. hydrophila* cytolytic enterotoxin (***AHcytoen***); cytotonic enterotoxins- heat-stable (***ast***) virulence genes were detected in 5; 3 and 5 out of 8 *A.hydrophila* studied strains, respectively (Fig. 1-5). In addition, specific gene of *Ps. aeruginosa* **(16SrDNA)**; outer membrane lipoprotein L (***opr*L**) and flagellin C (***fli*C**) genes were detected in all 8 studied *Ps. aeruginosa* strains; exotoxin A (***tox*A**) and exotoxin S (***exo*S**) virulence genes were detected in 5 and 6 out of 8 studied ones, respectively (Fig. 6- 10).

Regarding to *A. hydrophila* strains, they were identified genotypically through the detection specific gene of *A.hydrophila* (**16SrRNA**) and the PCR results showed that, it was amplified in all 8 studied *A. hydrophila* strains giving product of 625 bp. (Fig., 1) so, all of them were *A.hydrophila*. Similar detection was recorded by **Gordon *et al*. (2007)**; **Hussain *et al.* (2013); Skwor (2014)** and **Stratev *et al.* (2016).**

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* strains (Fig., 2) showed that, the ***aero*** gene was amplified in 5 out of 8 *A.hydrophila* studied strains giving product of 326 bp. Similar results were decided by **Singh *et al.* (2008)**; **Aravena *et al*. (2014)**; **Furmanek (2014)**; **Stratev *et al.* (2016)** and **Abd El Tawab *et al.* (2017)**. In addition, the results of PCR for amplification of ***AHcytoen*** gene in *A.hydrophila* strains (Fig., 3) showed that, the ***AHcytoen*** gene was amplified in 3 out of 8 *A.hydrophila* studied strains giving product of 232 bp. Similar results were recorded by **Sarkar *et al*. (2013); Cagatay and Şen (2014)** and **Abd El Tawab *et al.* (2017)** who reported that the detection of this cytolytic enterotoxin gene in such isolates and considered them a potential risk to human health. **Also,** the ***ast*** gene was amplified in 5 out of 8 *A.hydrophila* studied strains giving product of 331 bp. as shown in Fig. (4). Similar results were recorded by **Sha *et al.* (2002); Ashok *et al.* (2009); Bin Kingombe *et al*. (2010); Nawaz *et al.* (2010); Ye *et al.* (2013)** and **Abd El Tawab *et al.* (2017).** Moreover, the polar flagella (***fla***) gene is important for invasive ability; enterocyte adhesions and could be related to persistent or dysenteric presentation seen during Aeromonas infections and needed to be fully functional for optimal biofilm formation **(Ashok *et al*., 2009).** The results of PCR amplification of ***fla*** gene in *A. hydrophila* isolates showed that, the ***fla*** gene was amplified in all 8 studied *A. hydrophila* strains giving product of 608 bp. (Fig., 5). Similar findings were recorded by **Nawaz *et al.* (2010);** Aravena ***et al.*** **(2014)** and **Furmanek (2014).** Meanwhile, for *Ps. aeruginosa* strains, they also identified genotypically through the detection specific gene of *Ps. aeruginosa* (**16SrDNA**) and the PCR results showed that, it was amplified in all 8 studied *Ps. Aeruginosa* strains giving product of 956 bp. (Fig.,6) so, all of them were *Ps. aeruginosa*. Similar detection was recorded by **Spilker *et al*. (2004); Kumaran *et al*. (2010)**; **Uğur *et al*. (2012)**; **Abd El-Aziz (2015)** and **Abdel-Haq-Fatma El- Zahraa (2018).** The high virulence of *Ps. aeruginosa* is mostly due to its ability to produce a large number of virulence factors that can contribute to different ways to their pathogenicity **(Markey *et al*., 2013)**. The outer membrane lipoprotein L (***opr*L**) is a potent pro-necrosis factor for cells (**Picot *et al*., 2001**). It play important roles in the interaction of the bacterium with the environment as well as the inherent resistance of *Ps. aeruginosa* to antibiotics where the consequence of the presence of these specific outer membrane proteins that have been implicated in efflux transport systems that affect cell permeability **(Markey *et al*.,2013**). The obtained PCR result for amplification of ***opr*L** gene in *Ps. aeruginosa* strains revealed that, it was amplified in all 8 studied strains giving product of 504 bp. as shown in Fig. (7), as these protein is found only in this organism, it could be a reliable factor for rapid identification of *Ps. aeruginosa* in clinical samples (**De Vos *et al*., 1997**) so, all of them were *Ps. aeruginosa*. These results came in accordance with those recorded by **Xu *et al*. (2004); Lavenir *et al*. (2007); Abdullahi *et al*. (2013); Khattab- Mona *et al.* (2015) and Abd El Tawab *et al.* (2016).** The exotoxin S gene (***exo*S**) is toxic virulence factor causing death in the infected cell as it causes a decrease in DNA synthesis and viability

of host cells (**Kaufman *et al*., 2000).** The results of PCR for amplification of exotoxin S (***exo*S**) gene in *Ps. aeruginosa* isolates (Fig.,8) showed that, it was amplified in 6 out of 8 studied strains giving product of 118 bp. Similar findings were recorded by **Winstanley *et al*. (2005); Todar, (2011); Nikbin *et al*. (2012); Khattab- Mona *et al.* (2015) and Abd El Tawab *et al.* (2016).** In addition**,** exotoxin A (***tox*A**) is the most toxic virulence factor of the pathogenic *Ps. aeruginosa* for animals, fish and human (**Pollack, 2000** and **Michalska and Wolf, 2015**). The results of PCR amplification of exotoxin A (***tox*A**) gene (Fig., 9) revealed that, it was amplified in 5 out of 8 *Ps. aeruginosa* studied strains giving product of 396 bp. These results were agreed with those obtained by **Matar *et al*. (2002); Nafee (2012); Nikbin et *al*. (2012); Khattab- Mona *et al.* (2015) and Abd El Tawab *et al.* (2016).** Moreover, the results of PCR amplification of flagellin C (***fliC***) gene (Fig., 10) cleared that, it was amplified in all 8 *Ps. aeruginosa* studied strains giving product of 180 bp. These results came in harmony with those recorded by **Choi *et al*. (2002) and Ghadaksaz *et al*. (2015).**

Finally, most isolated *A. hydrophila*; *A. caviae; Ps. aeruginosa* and *Ps. fluorescens* strains were enterotoxigenic ones, as they had haemolytic; proteolytic; lipolytic; Lecithinase activities and had the ability for biofilm production, beside the detection of virulence genes in them In addition, virulence genes were detected in them indicates their pathogenicity for fish; a potential risk for causing fish - originated food poisoning and increasing prevalence of community-acquired infection is a dangers problem for public health.

References

1. Abd El Tawab, A. A.; Maarouf, A. A.; El Hofy, Fatma, I. and El - Mougy. E. A. (2017b): Detection of some virulence genes in *A. hydrophila* and *A. caviae* isolated from fresh water fishes at Qalubia Governorate. Benha Vet. Med. J., 33(2): 489-503.
2. Abd El Tawab, A. A.; Maarouf, A. A.; El Hofy, Fatma, I.; Salim, Amany, O. and El -Mougy. E. A. (2017a): Molecular studies on antibiotic resistant genes of Aeromonas species isolated from fish. Nature and Science., 15(12):90-97.
3. Abd El Tawab, A. A.; Maarouf, A. A. and Ahmed, Nesma, M. G. (2016): Detection of Virulence factors of Pseudomonas species isolated from fresh water fish by PCR. Benha Vet. Med. J., 30 (207.
4. Abd El-Aziz, D. M. (2015): Detection of Pseudomonas spp. in Chicken and Fish Sold in Markets of Assiut City, Egypt. J. Food Quality and Hazards Control., 2: 86-89.
5. Abdel-Haq, Fatma El- Zahraa, M. M. (2018): Control of pathogenic bacteria by antibacterial products produced by Lactic acid bacteria. Master Degree of Science in Botany (Microbiology) Zagazig University Faculty of Science.
6. Abdullahi, R.; Lihan, S.; Carlos, B. S.; Bilung, M. L.; Mikal, M. K.; and Collick, F. (2013): Detection of *oprL* gene and antibiotic resistance of *Pseudomonas aeruginosa* from aquaculture environment. European Journal of Experimental Biology., 3(6):148-152.
7. Alam, M. and Imran, M. (2018): Screening and Potential of Gram Negative Bacterial Isolates for their Extracellular Enzymatic Activities Isolated from the Hospital Aquatic Environment. J. Basic and Clinical Pharmacy., 9(1): 41-45.
8. Anguita, J.; Rodriguez-Aparicio, L. B. and Naharro, G. (1993): Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. Appl. Environ. Microbiology.,59: 2411-2417.
9. Aravena, R. M.; Inglis, T. J.; Riley, T. V. and Chang, B. J. (2014): Distribution of 13 virulence genes among clinical and environmental Aeromonas spp. in Western Australia. Eur. J. Clin. Microbiol. Infect. Dis., 33(11):1889-1895.
10. Ashok, K. C.; Joerg, G.; Horneman, J. A. and Johnson, J. A. (2009): Virulence factor–activity relationships (VFAR) with specific emphasis on Aeromonas species. J. water and health., 529- 554.
11. Austin, B. and Austin, D. A. (2007): Bacterial Fish Pathogens, Diseases of Farmed and Wild Fish Fourth Edition, Praxis Publishing Ltd, Chichester UK.
12. Banu, A.; Mathew, P.; Manasa, S. and Shetty, A. B. (2017): Bacteriological profile of burn wound infections with reference to biofilm formation. J. of Bacteriology and Mycology., 4 (4): 00101.
13. Bin Kingombe, C. I.; D'Aoust, J. Y.; Huys, G.; Lisa Hofmann, L. Rao,. M. and Kwan, J. (2010): Multiplex PCR method for detection of three Aeromonas enterotoxin genes. Applied and Environmental Microbiology., 76(2): 425–433.
14. Cagatay, I. T. and Şen, E. B. (2014): Detection of pathogenic Aeromonas hydrophila from rainbow trout (Oncorhynchus mykiss) farms in Turkey. Int. J. Agric. Biol., 16(2): 435–438.
15. Castro-Escarpulli, G.; Figuerasb, M. J.; Aguilera-Arreola, G.; Soler, L.; Ferna'ndez-Rendo'n, E.; Aparicio, G. O.; Guarro, J.; Chaco'n, M. R. (2003). Characterisation of Aeromonas spp. isolated from frozen fish intended For human consumption in Mexico, International Journal of Food Microbiology.,84 (2003) 41-49.
16. Choi, J. Y.; Sifri, C. D,; Goumnerov, B. C.; Laurence G. Rahme, L. G.; Ausubel, F. M. and Calderwood, S. B. (2002): Identification of virulence genes in a pathogenic strain of *Pseudomonas aeruginosa* by Representational Difference Analysis. J. Bacteriol., 184 (4):952-961.
17. Chopra, A. K. and Houston, C. W. (1999).

Enterotoxins in Aeromonas- associated gastroenteritis. Microbes Infect 1, 1129-1137.
18. Chu, W. H. and Lu, C. P. (2005): Multiplex PCR assay for the detection of pathogenic *Aeromonas hydrophila*. J. Fish Diseases., 28: 437- 441.
19. De Vos, D.; Lim, A.; Pirnay, J. P.; Struelens, M.; Vandenveld, C. (1997): Direct detection and identification of Pseudomonas aeruginosa in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane genes, oprI and oprL. J. Clin. Microbiol., 35: 1295-1299.
20. Eissa, N. M. E.; Abou El-Ghiet, E. N.; Shahen, A. A. and Abbas, A. (2010): Characterization of Pseudomonas Species Isolated from Tilapia" Oreochromis niloticus" in Qaroun and Wadi-El-Rayan Lakes. Egypt Global Veterinaria.,5 (2): 116-121.
21. El- Dien, W. M. S., Enas, M. S., Hanan, M. T. E., Salah, M. A. (2010):
22. prevalence of *Aeromonas hydrophila* and *Yarsinia entrocolitica* in some sea food sold In Sharkia Governorate markets and the effect of heat treatment on their viabilities Bulletin of animal health and production in Africa., 57 (3):191-197.
23. El-Hady, M. A. and Samy, A. A. (2011): Molecular Typing of Pseudomonas Species Isolated from Some Cultured Fishes in Egypt. Global Veterinaria., 7 (6): 576-580.
24. Furmanek, B. (2014): Phenotypic and molecular characteristics of an *Aeromonas hydrophila* strain isolated from the River Nile. Microbiol. Res., 169(7-8):547-552.
25. Ghadaksaz, A.; Fooladi; A. A. A.; Hosseini, H. H. and Amin, M. (2015): The prevalence of some Pseudomonas virulence genes related to biofilm formation and alginate production among clinical isolates. J. Applied Biomedicine., 13(1): 61-68.
26. Gonzalez-Serrano, C. J.; Santos, J. A.; Garcia-Lopez, M. L. and Otero, A. (2002): Virulence markers in *Aeromonas hydrophila and Aeromonas veronii biovar sobria* isolates from fresh water fish and from diarrhea case. J. Appl. Microbiol., 93 (3): 414-419.
27. Gordon, L.; Giraud, E.; Ganiêre, G. P.; Armand, F.; Bouju-Albert, A.; de la Cotte, N.; Mangion, C. and Le Bri, H. (2007): Antimicrobial resistance survey in a river receiving effluents from freshwater fish farms. J. Applied Microbiology., 102: 1167–1176.
28. Haas, M. J. (2001): Lipolytic microorganisms. In: F. P. Downes, K. Ito (eds.): Compendium of methods for the microbiological examination of foods 4th ed., American Public Health Association.,:175-182.
29. Hanna, M. I.; El-Hady, M. A.; Hanaa, A. A.; Elmeadawy, S. A. and Kenwy, A. M. (2014): Contribution on *Pseudomonas aeruginosa* infection in African Catfish (Clarias gariepinus). Research Journal of Pharmaceutical, Biological and Chemical Sciences., 5 (5): 575-588.
30. Hussain, I. A.; Geevaretnam, J.; Jeya, S. R.; Thirumalai, R. K. and Elango, J. (2013): Prevalence of hemolytic and enterotoxigenic Aeromonas spp. in healthy and diseased freshwater food fishes as assessed by Multiplex PCR. American J. Advanced Food Science and Technology., (1):70-85.
31. Ibrahim, Lamis, S. A. (2015): Studies on virulent and antibiotic resistant genes of Aeromonas species isolated from fish. Ph. D. (Bacteriology, Immunology and Mycology) Fac. Vet. Med. Suez Canal University.
32. Jvo, S. (2010): Pseudomonas a Communicative Bacteria. Product Manager Microbiology.,:2-4.
33. Kamel, G. M.; Ezz- Eldeen, N. A.; El-Mishad, M. Y. and Ezzat, R. F. (2011): Susceptibility Pattern of *Pseudomonas aeruginosa* Against Antimicrobial Agents and Some Plant Extracts with Focus on its Prevalence in Different Sources: Global Veterinaria., 6 (1): 61-72.
34. Kaufman, M. R.; Jia, J.; Zeng, L.; Ha, U.; Chow, M. and Jin, S. (2000): *Pseudomonas aeruginosa* mediated apoptosis requires the ADP-ribosylating activity of *exoS. J.* Microbiology.,146 (10): 2531-2541.
35. Khattab, M. A.; Nour, M. S. and El-Sheshtawy, N. M. (2015): Genetic Identification of *Pseudomonas aeruginosa* Virulence Genes among Different Isolates. J. Microbiol. Biochem. Technol., 7(5): 274-277.
36. Konemann, E.; Allen, S.; Janda, W.; Schreckenberger, C. and Winn, W. (1997): "Color Atlas and Textbook of Diagnostic Microbiology". Fifth Edition. Lippincott, Philadelphia, New York.
37. Kozinska, A. and Pekala, A. (2010): Serotyping of Aeromonas species isolated from polish fish farms in relation to species and virulence phenotype of the bacteria. Bull. Vet. Inst. Pulawy., 54: 315-320.
38. Kumaran, S.; Deivasigamani, B.; Alagappan, M.; Sakthivel, M. and Guru Prasad, S. (2010): Isolation and characterization of Pseudomonas species KUMS3 from Asian sea bass (Lates calcarifer) with fin rot. World J. Microbiol. Biotechnol., 26:359-363.
39. Lavenir, R.; Jocktane, D.; Laurent, F.; Nazaret, S. and Cournoyer, B. (2007): Improved reliability of *Pseudomonas aeruginosa* PCR detection by the use of the species specific *ecf X gene* target. J. Microbiol. Methods., 70: 20-29.
40. Maarouf, A. A.; Nassif, Marionette, Z.; Selim, Amany O. and Ali, Enas A. (2017): Bacteriological and molecular studies on Aeromonas organisms isolated from some species of fish and its public health hazards. Animal Health Research J., 5 (4 B): 549- 559.
41. Mankar, S. D and Barate, D. L. (2018): Isolation and Identification of amylase producing bacteria from soil receiving kitchen and agricultural waste. International J. Recent Scientific Research., 9 (1 H):

23408-23411.
42. Marcy, J. A. and Pruett, W. P. (2001): Proteolytic microorganisms. In:.
43. F. P. Downes, K. Ito (eds.): Compendium of methods for the microbiological examination of foods 4th ed., American Public Health Association.,: 183-186.
44. Markey, B. K.; Leonard, F. C.; Archambault, M.; Cullinane, A. and Maguire, D. (2013): Clinical Veterinary Microbiology. Second edition. MOSBY. Elsevier Ltd. Edinburgh London New York Oxford Philadelphia St Louis Sydney Toronto.
45. Matar, G. M.; Ramlawi, F.; Hijazi, N.; Khneisser, I. and Abdelnoor, A. M. (2002): Transcription Levels of *Pseudomonas aeruginosa exotoxin A Gene* and Severity of Symptoms in Patients with Otitis Externa. Current Microbiology., 45: 350–354.
46. Mavrodi, D. V.; Bonsall, R. F.; Delaney, S. M.; Soule, M. J.; Phillips, G. and Thomashow, L. S. (2001): Functional analysis of genes for biosynthesis of pyocyanine and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J. Bacteriol., 183(21): 6454-6465.
47. Meliani, A. and Bensoltane, A. (2015): Review of Pseudomonas Attachment and Biofilm Formation in Food Industry, Meliani and Bensoltane, Poult. Fish Wildl Sci., 2(3):1-8.
48. Michalska, M. and Wolf, P. (2015): Pseudomonas Exotoxin A: optimized by evolution for effective killing. Front. Microbiol., Department of Urology, Medical Center, University of Freiburg, Freiburg, Germany. J. Front. Microbiol.,6:1-7.
49. Nafee, S. K. (2012): Isolation and identification of clinical *Pseudomonas aeruginosa* producing exotoxin A and studying its toxic effect in mice, Thesis. M. V. Sc. College of Science/Baghdad Univ. Master of Science in Biotechnology.
50. Nawaz, M.; Khan, S. A.; Khan, A. A.; Sung, K.; Tran, Q.; Kerdahi, K. and Steele, R. (2010): Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. Food Microbiology., 27: 327-331.
51. Nikbin, V. S.; Aslani, M. M.; Sharafi, Z.; Hashemipour, M. and Shahcheraghi, F. (2012): Molecular identification and detection of virulence genes among *Pseudomonas aeruginosa* isolated from different infectious origins. Iran. J. Microbiol., 4: 118-123.
52. Omar, A.; Wright, J. B.; Schultz, G.; Burrell, R. and Nadworny, P. (2017). Microbial biofilms and chronic wounds. Journal of Microorganisms., 5(9): 1-15.
53. Palumo, S. A.; Maxono, F.; Willams, A. C.; Buchanan, R. L. and Thayer, D. W. (1985): Starch-ampicillin agar for the quantitative detection of *A. hydrophila.* J. Applied Environmental Microbiology., 50 (4):1027-1030.
54. Panda, S.; Bandyopadhyay, P. K. and Chatterjee, S. N. (2013): Characterization of *Pseudomonas aeruginosa* PB112 (JN996498) isolated from infected Labeo bata (Hamilton) by 16S rRNA gene sequence analysis and fatty acid methyl ester (FAME) analysis. African Journal of Biotechnology., 12(4): 400-405.
55. Parker, J. L. and Shaw, J. G. (2011): Aeromonas spp. clinical microbiology and disease. J. Infection., 62: 109-118.
56. Patil, U. and Chaudhairy, A. (2011): Optimal production of alkaline protease from solvent – tolerant alkalophillic *Pseudomonas aeruginosa* MTCC 7926. Indian. J. Biotechnol., 10: 329-339.
57. Pemberton, J. M., Kidd, S. P., Schmidt, R. (1997). Secreted enzymes of Aeromonas. FEMS Microbiol. Lett. 152., 1– 10.
58. Picot, L.; Abdelmoula, S.; Merieau, A.; Leroux, P.; Cazin, L.; Orange, N. and Feuilloley, M. G. J. (2001): *"Psudomonas fluorescens* as apotential pathogen: adherence to nerve cells " Microb. Infect., 3 (12): 985-995.
59. Pollack, M. (2000): *Pseudomonas aeruginosa.* In principle and practice of infection Diseases. Spp.2310-2335. Edited by G. L. Mandell; J.
60. E. Bennett and R, Dolin. Philadelphia: Churchill Livingstone.
61. Quinn, P. J.; Marky, B. k.; Carter, M. E.; Donnelly, W. J. C. and Leonard, F. C. (2002): Veterinary Microbiology and microbial diseases Blackwell scientific publications, Oxford, London.
62. Rabaan, A. A.; Gryllos, I.; Tomas, J. M. and Shaw, J. G. (2001): Motility and the polar flagellum are required for *Aeromonas caviae* adherence to HEp-2 cells. J. Infect. Immun., 69: 4257– 4267.
63. Roberts, R. J. (2001):"Fish pathology" 3rd Ed. Bailliere Tindall, London England.
64. Sakr, S. M. and Abd El- Rhman, A. M. M. (2008): Contribution on Pseudomonas septicemia caused by *Pseudomonas anguilliseptica* in cultured Oreochromus niloticus. 8th international symposium on Tilapia in Aquaculture Cairo International Convention Center, Egypt., 2: 1177-1197.
65. Sambrook, J.; Fritsch, E. F. and Montias, T. (1989): Molecular Biology. In: Molecular cloning. Laboratory manual, Second Edition. Cold Spring Harbor Laboratory press, USA.
66. Sarkar, A.; Saha, M. and Roy, P. (2013): Detection of 232bp virulent gene of pathogenic *Aeromonas hydrophila* through PCR Based Technique: (A Rapid Molecular Diagnostic Approach). Advances in Microbiology., 3(1):1-5.
67. Sha, J. E.; Kozlova, V. and Chopra, A. K. (2002): Role of various enterotoxins in *A. hydrophila*-induced gastroenteritis: Generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. J. Infect. Immun., 70(4): 1924–1935.
68. Shayo, S. D.; Mwita, C. J. and Hosea, K. M. (2012): Virulence of Pseudomonas and Aeromonas bacteria

recovered from Oreochromis niloticus (Perege) from Mtera hydropower Dam Tanzania Annals of Biological Research., 3 (11):5157-5161.
69. Singh, V., Rathore, G., Kapoor, D., Mishra, B. N. and Lakra, W. S. (2008): Detection of aerolysin gene in *Aeromonas hydrophila* isolated from fish and pond water. Indian J. Microbiol., 48:453– 458.
70. Skwor, T.; Shinko, J.; Augustyniak, A.; Gee, C. and Andraso, G. (2014): *A. hydrophila* and *A. veronii* predominate among potentially pathogenic ciprofloxacin- and tetracycline-resistant Aeromonas isolates from Lake Erie. J. Appl. Environ. Microbiol., 80(3):841-848.
71. Spilker, T.; Coenye, T.; Vandamme, P. and Lipuma, J. (2004): PCR- Based assay for differentiation of *Pseudomonas aeruginosa* from other Pseudomonas species recovered from cystic fibrosis patients. J. Clin. Microbiol., 42(5):2074-2079.
72. Sreedharan, K.; Philip, R. and Singh, I. S. (2012): Virulence potential and antibiotic susceptibility pattern of motile Aeromonads associated with freshwater Ornamental fish culture systems: A possible threat to public health. Brazilian J. Microbiology.,: 754- 765.
73. Stoyanova, V.; Georgieva, S.; Iliev, I.; Marhova, M. and Trifonova, S. (2012): Lipolytic activity of genus Pseudomonas. J. BioSci. Biotech., SE/ONLINE.,: 163-168.
74. Stratev, D.; Daskalov, H. and Vashin, I. (2015): Characterization and determination of antimicrobial resistance of β -haemolytic Aeromonas spp. isolated from common carp (*Cyprinus carpio* L.). Revue Méd. Vét., 166(1-2): 54-61.
75. Stratev, D.; Gurova, E.; Vashin, I. and Daskalov, H. (2016): Multiplex PCR detection of haemolysin genes in β-haemolytic Aeromonas hydrophila strains isolated from fish and fish products. Bulgarian J. Agricultural Science., 22 (2): 308–314.
76. Thayumanavan, T.; Vivekanandhan, G.; Savithamani, K.; Subashkumar, R. and Lakshmanaperumalsamy, P. (2003): Incidence of haemolysin -positive and drug resistance *Aeromonas hydrophila* in freshly caught fmfish and prawn collected from major commercial fish of Costal South India. FEMS. Immunol. and Med. Microbiol., 6 (1/2): 41-45.
77. Todar, K. (2011):"Todar's Online Textbook of Bacteriology" http: \\ www. textbookofbacteriology. net.
78. Tole, S. B.; Fawade, M. M. and Patil, S. M. (2016): Amylase activity of a starch degrading bacteria isolated from soil around Tulja- Bhavani Temple of Tuljapur. IOSR J. Pharmacy and Biological Sciences., 11(6 Ver. II): 01-06.
79. Uğur, A. ; Ceylan, O. and Ashm, B. (2012): Characterization of Pseudomonas spp. from seawater of the southwest coast of Turkey. J. Biol. Environ. Sci., 6(16): 15-23.
80. Winstanley, C.; Kaye, S. B.; Neal, T. J.; Chilton, H. J.; Miksch, S.; Hart, C. A. and the Microbiology Ophthalmic Group (2005): Genotypic and phenotypic characteristics of *Pseudomonas aeruginosa* isolates associated with ulcerative keratitis. J. Med. Microbiol., 54:519–526.
81. Xu, J.; Moore, J. E.; Murphy, P. G.; Millar, B. C. and Elborn, J. S. (2004): Early detection of *Pseudomonas aeruginosa* - comparison of conventional versus molecular (PCR) detection directly from adult patients with cystic fibrosis (CF). Annals of Clinical Microbiology and Antimicrobials., 3:21.
82. Ye, L.; Lu, Z.; Li, X.; Shi, L.; Huang, Y. and Wang, H. H. (2013). Anti bioticresistant bacteria associated with retail aquaculture products from Guangzhou, China. J. Food Prot., 76, 295- 301. doi:10.4315/0362-028X. JFP-12-288.
83. Zhu-Da Ling; Li-Ai Hua; Wang-Jian Guo; Li-Ming; Cai-Tao Zhen; Hu-Ging (2006): "The correlation between the distribution pattern of virulence genes and the virulence of *Aeromonas hydrophila* strains." Acta-Scientiarum-Naturalium-Universitatis-Sunyatseni., 45(1): 82-85.
84. Zilberberg, M. D. and Shorr, A. F. (2009): Epidemiology of healthcare- associated pneumonia (HCAP). Semin. Respir. Crit. Care Med., 30:10–15.

10/5/2019