Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(6): 453 – 467 (2020)
www.ejabf.journals.ekb.eg

Phenotypic and Genotypic characterization of the pathogenic *Pseudomonas aeruginosa* isolated from cultured *Pangasianodon hypophthalmus* in Egypt.

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ARTICLE INFO

Article History:
Received: Aug. 29, 2020
Accepted:Sept.30, 2020
Online: Oct. 3, 2020

Keywords:
P. hypophthalmus,
P. aeruginosa
VITEK 2,
16S rDNA gene
Virulent genes.

ABSTRACT

This study was conducted on 70 diseased *Pangasius hypophthalmus* fish collected from different *Pangasius* fish farms in Egypt. Bacteriological examination revealed the isolation of *P. aeruginosa* that was biochemically confirmed using MALDI-TOF MS and VITEK 2 compact system. The prevalence and virulence of *Pseudomonas aeruginosa* were estimated genotypically using species specific 16S rDNA gene, *OprL* and *ToxA* virulent genes for detection of pathogenic strains followed by sequencing analysis. Pathogenicity of isolated *Pseudomonas* aeruginosa was confirmed by experimental intraperitoneal injection of healthy *Pangasius hypophthalmus* with positively virulent strains resulting in high mortalities among injected fish.

INTRODUCTION

Pangasius hypophthalmus (Sauvage, 1878) is an omnivorous fresh water fish originating from the Mekong River in Vietnam (**Rodríguez *et al.*, 2018)**. Panga has several names as sutchi catfish, striped catfish, iridescent shark-catfish, or tra fish, it has high acceptability by consumers due to its low price, good taste and white meat that can replace cod or other expensive white fishes **(Little *et al.*, 2012 ; Guimarães *et al.*, 2016).**

Recently, *Pangasius* become one of the best aquaculture candidates in several countries as it can be cultured easily in ponds, concrete tanks or fish cages, it has the ability to survive at high stocking density, poor water quality, low oxygen levels and can easily accept external feeding. Moreover, it considered as a fast growing fish with high yield production and disease resistant that meet up the farmer's demand. **(Hill and Hill, 1994; Ali and Haque, 2011; Vaishnav *et al.*, 2017).**

Bacterial fish diseases are the main cause of high mortalities with disastrous economic losses among several fish species in different cultured conditions **(Austin and Austin, 2007).** Several reports mentioned *Aeromonas hydrophila* and *Edwardsiella ictaluri* as the most significant bacterial problems affecting *Pangasius* species **(Crumlish *et al.*, 2002; Faruk, 2008).** *Pseudomonas*, also known as ulcerous disease, is a common fish disease caused by several members of family Pseudomonadaceae as *P. fluorescens, P. angulliseptic, P. diminuta, P. aeruginosa* and *P. putida* **(Markey *et al.*, 2013)**, where *P. fluorescens, and P. aeruginosa* are the most common opportunistic pathogens affecting fishes in aquaculture (**Ndi and Barton, 2012).** *Pseudomonas aurginosa* is a gram-negative, rod shaped obligatory aerobic organism that can infect fishes especially under stressful environmental conditions causing ulcer type diseases including ulcerative syndrome, Pseudomonas septicemia **(Wiklund and Bylund, 1990; El-Nagar, 2010; Abdullahi *et al.*, 2013)**. Moreover, *Pseudomonas aurginosa* can cause life-threatening infections in immunocompromised individuals and is the main cause of nosocomial infections **(Van Delden and Iglewski, 1998; Ndi and Barton, 2012)**

Pseudomonas aeruginosa has several virulence factors that may contribute to its pathogenicity via attachment, colonization, termination, and extent through host tissue, interruption of tissue components and cytotoxicity such as exotoxin A, exotoxin U, exotoxin S , nan 1 genes, elastase (*lasB*), alginate (*algD*), hemolytic phospholipase C (*plcH*), flagellar filament structural protein (*fliC*), also the outer membrane proteins; *OprI* and peptidoglycan-associated lipoprotein *OprL*; that play important roles in the interaction of the bacterium with the environment as well as involved in the efflux transport systems that affect cell permeability **(Khattab *et al.*, 2015; Fadhil *et al.*, 2016)**

Few literature discussed *Pseudomonas aeruginosa* infection in *Pangasius* fish. So, the aim of the current study was isolation and phenotypic; molecular identification of *Pseudomonas aeruginosa* isolates reterived from naturally infected *Pangasius* fish collected from different localities during the harvest season with detection of some virulent genes in the isolated strains.

MATERIALS AND METHODS

Fish sample:

A total number of 70 naturally infected *Pangasius* fish (60.5 ±9 gm) were collected from European countryside fish farms and private fish farms in Kafr El Sheikh Governorate. The water source was underground water and drainage; sewage water respectively. The collected fish were subjected to clinical and postmortem examination for recording any abnormalities.

Bacteriological Isolation and Phenotypic Identification of *P. aeruginosa*:

The collected samples (liver, kidney, gills and spleen) underwent the standard microbiological examination under complete aseptic conditions. The selected organs

were inoculated on nutrient broth (Oxoid, England) and incubated at 37 °C then sub cultured on nutrient agar (Oxoid, England) and Pseudomonas agar base (Oxoid, England) under aerobic condition and incubated at 37 °C. The isolated bacterial colonies were purified according to the basis of their morphological characters and subjected to phenotypic identification using Matrix Assisted Laser Desorption Time of Flight Mass Spectrometry (**MALDI-TOF MS)** (Biomerieux, france) and VITEK 2 compact system (Biomerieux, france) **(Karthiga *et al*., 2016).**

Molecular Identification And Sequencing Analysis:

DNA extraction from biochemically confirmed isolates performed by QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturers instructions. 16S rDNA gene was used to confirm the isolation of *P. aeruginosa*. *OprL* and *ToxA* virulent genes were used for detection of bacterial virulence. The oligonucleotide sequences of used primers are shown in **Table 1**. PCR reaction was performed in 50-µl total volume consisting of 25-µl Maxima Hot Start Master Mix (2X) (Thermo), 1 µl (20 uM) for each forward and reverse primers, 5 µl Template DNA and PCR grade water up to 50 µl.The cycling condition of the used primers are shown in **Table 2**. PCR products were loaded on 1% agarose gel stained with Ethidium bromide (10 mg/ml) in Tris Acetate EDTA buffer (TAE) against 1Kb plus ladder (Fermentas) by electrophoresis and visualized with UV Trans-illuminator. Genetic Analyzer 3500 sequencer (Applied Bio systems) was used for Sequencing analysis of bacterial isolates and the results were aligned against those in database of the GenBank (National Center for Biotechnology Information ‘‘NCBI”, Bethesda, MD, USA) using BLAST**.**

Table 1.: Nucleotide primer sequences of *P. aeruginosa* Target genes

Target Genes	Primer sequence	Product size	Reference
16SrDNA	F:GGGGGATCTTCGGACCTCA	956bp.	**Abu-Elala, *et al*., 2016**
	R:TCCTTAGAGTGCCCACCCG		
ToxA	F:GGT AAC CAG CTC AGC CAC AT	352bp.	**Wendt *et al*., 2017**
	R:TGA TGT CCA GGT CAT GCT TC		
OprL	F: ATG GAA ATG CTG AAA TTC GGC	504bp.	**Xu *et al*., 2004**
	R: CCT CTT CAG CTC GAC GCGACC		

Table 2.: Thermocycling conditions of used primers.

Target Genes	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
16SrDNA gene (25 cycle)	95 °C for 2 min	95 °C for 20 sec	58 °C for 20 sec	72 °C for 40 sec	72 °C for 5 min
***ToxA* gene (30 cycle)**	94 °C for 3 min	94 °C for 30 sec	55 °C for 1 min	72 °C for 1min	72 °C for 5 min
***OprL* gene (40 cycle)**	96 °C for 5 min	96 °C for 1 min	55 °C for 1 min	72 C for 1min	72 C for 10 min

Pathogenicity test:

Sixty apparently healthy *Pangasianodon hypophthalmus* with an average body weight 70 ± 9 g were obtained from *Pangasius* fish hatchery in Kafr El Sheikh governorate, Egypt for experimental challenge. Fish fed on basal diet; 3% of their body weight twice daily. Water was exchanged weekly to maintain optimum water quality. The aquaria were supplied with sufficient chlorine free tap water, aeration was carried by electric aerator. The temperature and pH were adjusted at 30 ± 2 C and 7.4 using water electric heater and PH meter respectively. Fish were acclimated for two weeks before experimental infection, the apparently healthy fish were divided in glass aquaria (80 X 40 X 30 cm) into 2 groups in duplicates (15 fish per group) first group was injected intraperitoneally with one isolated virulent strain of *P.aeruginosa* (positive to 16SrDNA gene, ToxA and OprL virulent genes) at a dose of 0.2 ml of 3×10^7 CFU (**Eissa *et al*., 2010).** Second Control group were I/P injected with sterile saline. Clinical signs and mortalities were observed and recorded daily for a week. The institutional Animal Care and Use Committee (IACUC) has approved the protocol of the experiment (IACUC protocol No.Vet CU16072020186).

Histopathological examination:

Tissue specimens from gills, liver, kidneys, and spleen were taken from experimentally infected *Pangasius hypophthalmus* at the seventh day post infection. Samples were fixed in 10% formalin, processed by conventional method, sectioned at 4 μm and stained with Hematoxylin and Eosin **(Roberts, 2001).**

RESULTS

1- Clinical and Postmortem findings:

The examined fish showed hemorrhages especially at the ventral part of abdomen, base of the fins and mouth. Some fish showed eye opacity, tail and fins rot. Internal examination showed abdominal dropsy, paleness and enlarged liver; congested kidneys; in addition to congested and enlarged spleen.

2- Bacteriological Isolation and Phenotypic Identification of *P. aeruginosa*:

Microbiological examination revealed the isolation of *Pseudomonas aeruginosa* where the growing colonies were large and irregular with blue green pigmentation on Pseudomonas agar base media**.** Biochemically, *P.aeruginosa* purified colonies were tested for oxidase, catalase, H_2S production, Lipase test and lysine decarboxylase (LDC) positive but citrate, urease and Ornithine decarboxylase (ODC) test negative and resistant to vibrio static agent O/129 by VITEK 2 compact system as shown in **Table 3.**

3- Prevalence of *Pseudomonas aeruginosa* infection :

The prevalence of *P.aeruginosa* was 71.42% from total number of infected fish; it was isolated from 18 liver samples; 14 kidney samples; 11 gill samples and 7 spleen samples with prevalence of 36%, 28%, 22% and 14% respectively as shown in **Table 4 and 5.**

Table 3. VITEK 2 compact system Biochemical profile of *Pseudomonas aeruginosa*

2	APPA	+	3	ADO	_	4	PYrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
١٠	H2S	+	11	BANG	+	12	AGLTP	+	١٣	dGLU	+	١٤	GGT	_	١٥	OFF	+
١٧	BGLU	_	18	DMAL	+	١٩	dMAN	+	20	dMNE	+	21	BXYL	_	٢٢	BALaP	_
٢٣	ProA	+	26	LIP	+	٢٧	PLE	_	29	TyrA	+	31	URE	_	32	Dsor	_
٣٣	SAC	+	34	DTAG	_	٣٥	DTRE	+	٣٦	CIT	_	37	MNT	_	39	SKG	-
٤٠	ILATK	+	41	AGLU	_	٤٢	SUCT	+	٤٣	NAGA	_	44	AGAL	-	45	PHOS	+
٤٦	GLYA	_	47	ODC	-	٤٨	LDC	+	٥٣	IHISa	_	56	CMT	_	57	BGUR	_
٥٨	O129R	+	59	GGAA	+	٦١	IMLTa	+	٦٢	ELLM	+	٤٦	ILATa	+			

Table (4). Prevalence of *P. aeruginosa* infection in different fish farms in *P.hypophthalmus* fish.

Pangasius farms	Number of collected fish	Number of infected fish	% of infection*
European countryside fish farms	35	20	40%
Private fish farms in Kafr El Sheikh	35	30	60%
Total	70	50	71.42%

* % of infection from total number of infected fish

Table (5): Prevalence of isolation of *P. aeruginosa* from different organs

Fish farms / organs	European countryside fish farms		Private fish farms in Kafr El Sheikh		Total	
	No	%	No	%	No	%
Liver	8	40%	10	33.3%	18	36%
Kidney	6	30%	8	26.7%	14	28%
Gills	4	20%	7	23.3%	11	22%
Spleen	2	10%	5	16.7%	7	14%

No.: Number of isolates. %: Percentage in relation to total number of isolates

4- Molecular Identification and Sequencing Analysis:

16S rDNA gene produce positive amplicons in all detected *P.aeruginosa* isolates at 956 bp. (**Fig. 1A).** Virulent *ToxA* and *OprL* gene produce amplified products at 352 bp. and 504 bp. respectively. (**Fig. 1B, 1C).** Sequencing results of positively virulent strains of *P.aeruginosa were* blasted against highly identical sequences present on gene bank and showed high identity percentage (99%) and registered on gene bank with accession number MT006363.

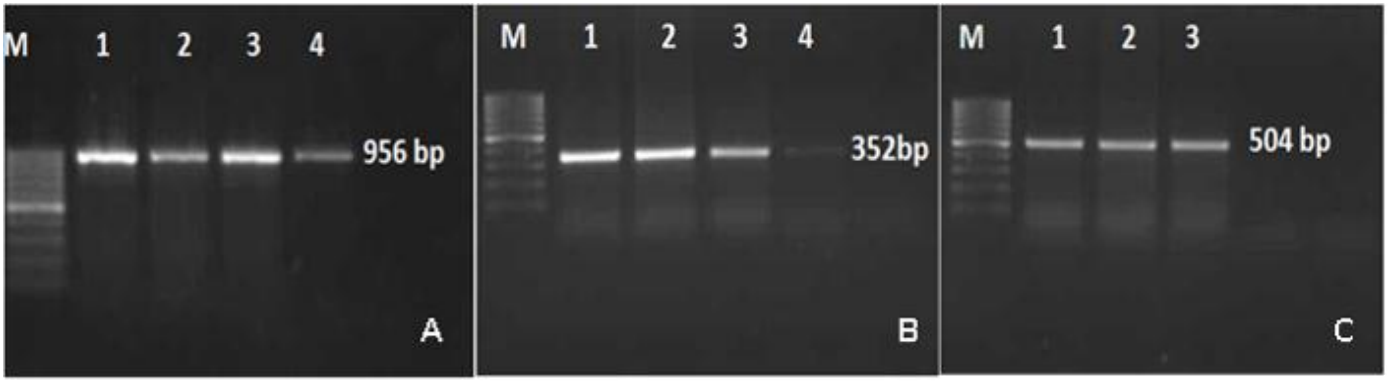

Fig. 1 PCR products on agarose gel electrophoresis appeared as (A) positive amplicons of 16srDNA gene of *P.aeruginosa* at 956bp. (B) *ToxA* virulent gene at 352 bp. and (C) *OprL* positive virulent gene at 504bp.

5- Pathogenicity test:

After 7 days of fish observation The infectivity test revealed that the mortality rate in I/P infected *Pangasius* fishes with *P. aeruginosa* representing 90% of the total number of fishes. The clinical signs and post mortem lesions were similar to those described during the natural infection.

6-Histopathological examination:

The Histopathological changes observed in infected *P.hypophthalmus* fish were focal areas of fused secondary gill lamellae and some areas of completely necrosed gill lamellae , together with hemorrhage in many gill arches. **Fig. 2A, 2B.** The intestine showed submucosal lymphoid depletion, blood vessel dilatation and congestion **Fig. 2C, 2D.** Areas of hemorrhage, leukocytic cells infiltrations and mononuclear cells infiltrations were obvious in renal interstitial tissue with diffuse necrosis in the renal tubules **Fig. (2E, 2F).** Spleen white pulp showed depletion and necrosis **Fig. 2G, 2H** and Hepatocytes had congested and dilated blood vessels. **Fig. 2I, 2J.** No pathological changes were observed in the liver and kidney of control fish.

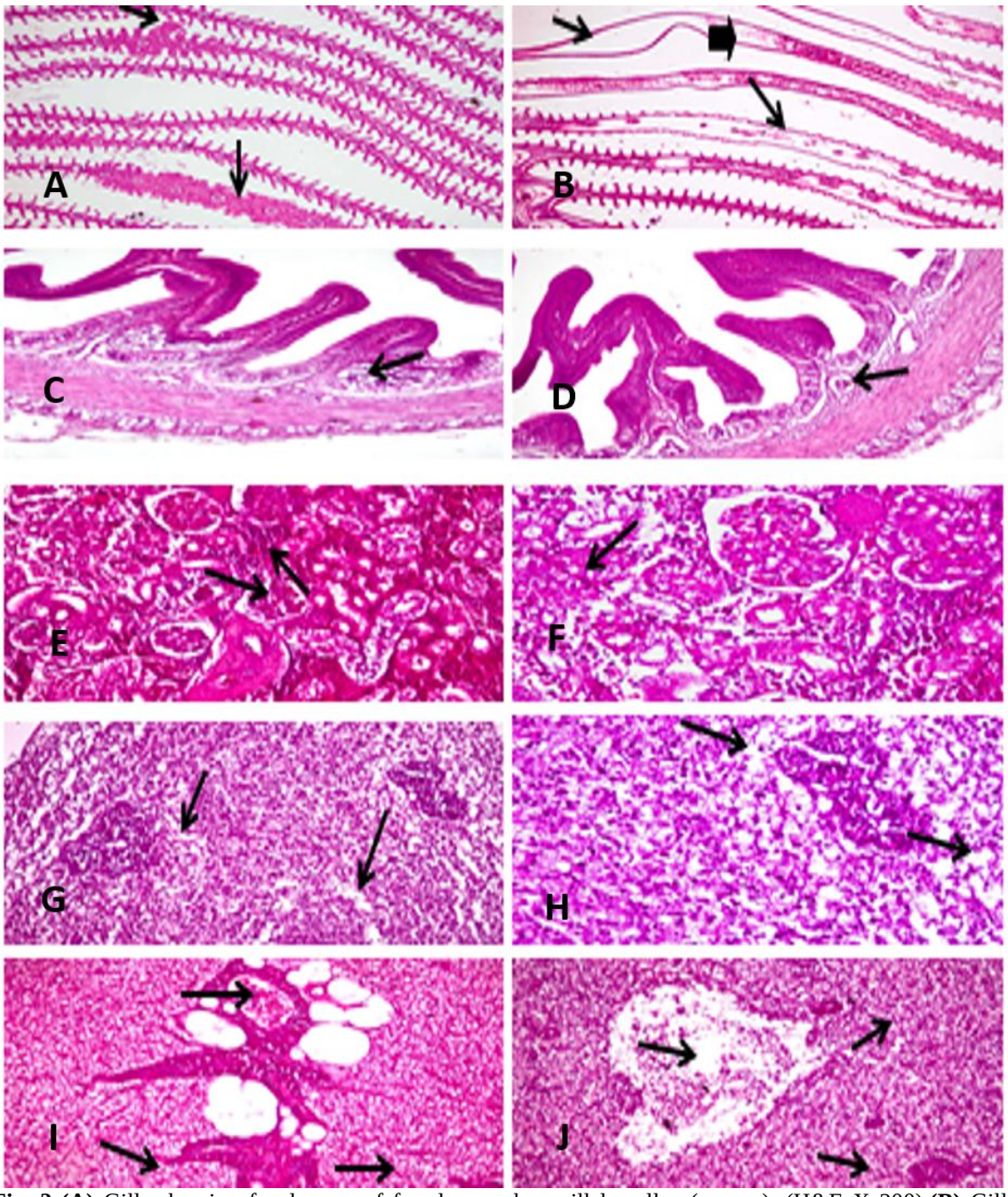

Fig. 2 (A) Gills showing focal areas of fused secondary gill lamellae (arrows), (H&E X 200).**(B)** Gills showing some areas of completely necrosed gill lamellae (arrows), together with hemorrhage in many gill arches (arrow head), (H&E X 200). **(C)** Intestine showing submucosal lymphoid deplesion (arrow), (H&E X 200**).((D)** Intestine showing submucosal blood vessel dilatation and congestion (arrow), (H&E X 200**).(E)** Kidneys showing interstitial tissue areas of hemorrhage (arrow head), and leucocytic cells infiltrations (arrows), (H&E X 200**).(F)** Kidneys showing diffuse necrosis in the renal tubules (arrows) and interstitial tissue mononuclear cells infiltrations (arrow head), (H&E X 400).**(G)** Spleen showing depletion and necrosis of the white pulp (arrows), (H&E X 200).**(H)**Spleen showing depletion and necrosis of the white pulp with higher magnification (arrows), (H&E X 400).**(I)** Liver showing congestion of the blood vessel (arrow head) and diffuse hepatocytes vacuolation (arrows), (H&E X 200).**(J)**Liver showing dilatation of the blood vessel (arrow head) and diffuse hepatocytes vacuolation (arrows), (H&E X 400).

DISCUSSION

Pseudomonas aeruginosa is one of the primary global causes of septicemia in both fresh and marine water fishes with severe economic losses in fish farms **(Austin and Austin, 1999 and Roberts, 2001)**. This study may be the first report on isolation and characterization of *Pseudomonas areuginosa* from *Pangasius hypophthalmus* fish in Egypt.

The observed clinical signs in infected *Pangasius* were similar to that reported by **Khalil *et al.*, (2010); Younes *et al.*, (2015) and Elgohary *et al.*, (2020)** where the infected fishes showed hemorrhages, tail rot, ulcer on the skin, dark pigmentation and abdominal distention. The post mortem findings is in concordance with **Altinok *et al.*, (2007); Sakr and Abd El- Rhman, (2008)** and **Elham *et al.*, (2017)** that liver was pale while spleen and kidney were congested and enlarged. This septicemic picture may be attributed to cytotoxins and other extracellular products produced by *Pseudomonas aeruginosa* (**Todar, 2010)**

Isolated *Pseudomonas aeruginosa* appeared as gram negative rods, with blue green pigmentation on Pseudomonas agar base media, this color resulted from the production of water soluble pyocyanin pigment: a blue green phenazine compound; which considered the most characteristic feature of *Pseudomonas aeruginosa* that distinguish it from other *pseudomonas* spp. (*P.fluorescens, P.putida, P.stutzeri, P.putrefa*). **(El-Fouly *et al.*, 2014; Abdul-Hussein and Atia, 2016).**

Phenotypic identification tools as Matrix Assisted Laser Desorption Time of Flight Mass Spectrometry (MALDI-TOF MS) **(Dupont *et al.*, 2010)** and VITEK 2 compact system are still the most valuable testing tools for accurate diagnosis of typical isolates of *P.aeruginosa* (**Garcia-Garrote *et al.*, 2000; Bruins *et al.*, 2004).** *Pseudomonas aeruginosa* were positively reacted to oxidase, catalase and H_2S production in agreement with **Hossain *et al.*, (2006)** and **Akinyemi, (2012).** Nonetheless**,** negatively reacted to citrate utilization and urease in contrast with **Musa *et al.*, (2009)** and **Hamzah, (2018).** This failure in biochemical assimilation may be attributed to usage of small inoculum during sample analysis by VITEK 2 system or the occurrence of bacterial divergence where the bacterial species are genetically identical, but with different phenotypic characteristics. (**Ravea *et al.*, 2019).**

The prevalence of *P. aeruginosa* was 71.42% of total number of infected fish nearly similar to **Elham *et al.*, (2017)** who recorded isolation of *P. aeruginosa* with prevalence rate 60 % but higher than that recorded by **Eissa *et al.*, (2010); EL-Hady and Samy, (2011) and Hanna *et al.*, (2014)** where prevalence of infection with *P.aeruginosa* were 30.83% and 13.3% from naturally infected *Oreochromis niloticus* respectively; 45.5% in *Cyprinus carpio* and an average of 32.3 % in *Oreochromis niloticus*, *Cyprinus carpio* and *Clarias gariepinus* respectively. The highest prevalence percentage of *P.aeruginosa* was in order from liver, kidney, gills and lowest percentage was recorded from spleen samples

as recorded by **Atwa, (2007); Sakr and Abd El- Rhman, (2008)** and **Elham *et al.*, (2017)** who isolated *P. aeruginosa* from liver of *O. niloticus* and *Tilapia zilli* with intensity of 75% and 72.7% respectively. The difference in prevalence percentage may be related to fish species, location and environmental conditions.

Species specific 16SrDNA act as a preliminary gene for detection of *P.aeruginosa* as it provides unambiguous data even for rare isolates, **(Hossain *et al.*, 2006** ; **Abu-Elala *et al.*, 2016).** Our PCR results revealed the detection of this gene from all isolated *P.aeruginosa* strains at 956 bp. The Virulence ability of *P.aeruginosa* were confirmed via detection of Exotoxin A encoded by the *toxA* gene at 352bp. ;similarly to **Nafee, (2012); Rana *et al.*, (2017)** and **Wendt *et al.*, (2017),** this gene posses its pathogenicity by inhibiting host protein biosynthesis. **(Jaffar-Bandjee *et al.*, 1995; Hamood *et al.*, 2004).** The external Outer membrane lipoprotein *(oprL*) produce positive amplicons at 504 bp. in accordance with **Nowroozi *et al.*, (2012); Markey *et al.*, (2013) and Abdullahi *et al.*, (2013)**, this protein responsible for antibiotics resistance and could be the only reliable factor for rapid identification of *P.aeruginosa*. **(Oluwole and Chinyere, 2012; khattab *et al.*, 2015).**

Concerning the pathogenicity test, the intraperitoneally injected fishes showed clinical signs and post mortem lesions similar to those described during the natural infection with high mortality rate as that reported by **Elham *et al.*, (2017)** who reported 80% mortality in the experimentally infected *O. niloticus* fishes with *P. aeruginosa*. Mortalities could be explained by the expression of cell-associated virulence factors such as pili, flagella, lipopolysaccharide and a number of extracellular products such as protein exotoxin A, proteases, haemolysin with lecithinase activity, elastase (Las B and Las A), siderophores (pyochelin, pyocyanin, and pyoverdin) and phospholipase C **(Delden and Iglewski, 1998; Mavrodi *et al*., 2001; Markey *et al*., 2013)**.

Histopathological changes were completely necrosed gill lamellae, together with hemorrhage in many gill arches, hypertrophy and hyperplasia of the epithelial cells lining the secondary lamellae resulting in lamellar fusion were observed similar to that reported by **Devakumar *et al.*, (2013)** and **Thomas *et al.*, (2014)** in gills of *P.aeruginosa* infected crabs and *Oreochromis mossambicus* fish respectively. The intestinal histopathological lesions were in accordance with **Hanna *et al.*, (2014)** that intestine showed submucosal lymphoid deplesion, hypertrophy and submucosal blood vessel dilatation and congestion.

The Kidneys showed diffuse necrosis in the renal tubules and widespread tubular degeneration similar to findings observed by **Amosu, (2012); Hanna *et al*., (2014) and Elgohary *et al.*, (2020)** in African Catfish,(*Clarias gariepinus*) inoculated with different concentrations of *Pseudomonas aeruginosa and* naturally infected *O.niloticus* in Fayoum governorate respectively. This changes consequently lead to renal dysfunction. Moreover, The Spleen showed depletion and necrosis of the white pulp and the Liver showed congestion of the blood vessels and diffuse hepatocytes vacuolation similar to the

findings of **Kumaran *et al.*, (2010); Oh *et al.*, (2019) and Elgohary *et al.*, (2020)** in *Pseudomonas* infected Sea bass; rainbow trout and *O. niloticus* fish respectively.This seveer tissue damage is highly correlated with exotoxins secreted by *P. aeruginosa* (**Iglewski *et al.*, 1977)**.

In conclusion, Septicemic clinical signs appeared on cultured *Pangasianodon hypophthalmus* fish was accredited to pathogenic *Pseudomonas aeruginosa*, that was Phenotypically and Molecularly confirmed via detection of 16SrDNA and *ToxA*;*OprL* virulent genes.

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