Prevalence, Molecular Characterization, Pathogenecity and Antimicrobial susceptibility of *Pseudomonas fluorescens* isolated from *Oreochromis niloticus*

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Abstract: *Pseudomonas fluorescens*is responsible for high mortalities in *Oreochromis niloticus* in Egypt. It is one of the most important fish pathogens infecting both fresh and brackish water fish species; containing virulence genes that enhance bacterial pathogenesis in the host. The current study aimed to isolate, identify and characterize *Ps. Fluorescens* and determination of the seasonal prevale ncein *O. niloticus* in Kafr El-Sheikh governorate, Egypt, using selective differential medium (Pseudomonas–Fagar), morphological and biochemical tests. Pathogenesis of *Ps. fluorescens* was checked by experimental infection of *O. niloticus* together with detection of the extracellular alkaline metalloprotease virulence gene *(aprX*). The seasonal prevalence of the isolated *Ps. Fluorescens* strains, revealed that the highest incidence was during the winter season (41.2%), autumn (25.5%), spring (19.6%) and summer (13.7%), respectively. The PCR revealed the presence of the virulence gene (aprX) in the selected *Ps. Fluorescens* strain which has an important role in the pathogenicity. *Ps. fluorescens* strains are highly sensitive to Amikacin (96.1%) and highly resistant to Ampicillin and Streptomycin (100%). Moreover, the histopathological studies revealed severe changes in the experimentally infected fish tissues.
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Key words*:* Antibiogram, Aprx, Pathogenecity, *Pseudomonas fluoroscens*, Freshwater fish

1. Introduction

Aquaculture intensification is a world scoop, that gradually developed in the world as well as in Egypt, to face the increasing demand of protein for human consumption (**Asaad, 2008**). *Oreochromis niloticus* has attained a great economic importance in Egypt. Infectious diseases of cultured fish are the most impediments on the expansion and development of aquaculture (**Younes and Gaafar, 2014**).

Bacterial diseases are the major problems in the intensive aquaculture (**Ibrahim *et al.*, 2013**). Bacterial fish pathogens are naturally present in the fish surrounding environment; however, under certain stress conditions, may cause severe economic losses with 80% of mortalities in fish farms. *Pseudomonas* infection has been incriminated as one of the most common bacterial infection among fish and appear to be stress related disease of freshwater fish, especially under culture conditions **(Khalil *et al.*, 2010)**.

Pseudomonas fluorescens; a dominant component of freshwater ecosystem; has been considered as a fish spoilage organism as well as a primary, but poor pathogen. *Ps. fluorescens*is normally found in water, soil and on the body of fishes (**Darak and Barde, 2015)**.

Pseudomoniasis; the disease caused by *Pseudomonas fluorescens*, is usually associated with septicemia and ulcerative conditions in a wide range of fishes and characterized by petechial hemorrhage, darkness of the skin, detachment fscales, abdominal ascites and exophthalmia. It occurs throughout the year, especially at low temperatures. (**Swain *et al.*, 2007**). *Ps. fluorescens* is a gram-negative, rod shape organism, motileby polar flagella, able to grow between 25 to 30°Cbut not at 40°C and has the ability to produce fluorescent pigment (fluorescein) **(Darak and Barde, 2015).**

For pathogenic bacteria, secreted extracellular metalloproteases often play important roles during infection. This may be attributed to its utilization by the bacteria for mucosal colonization and interaction with hosts (**Denkin and Nelson, 2004; Valiente *et al.*, 2008**). The virulence mechanism of aquatic *Ps. fluorescens* remains largely unknown; many strains are able to secrete an extracellular protease called AprX. However, the involvement of extracellular

metalloproteases in the virulence of the pathogenic bacteria to fish is not a general rule; in contrast, the AprXof *Ps. fluorescens* (an extracellular alkaline metalloprotease of the serralysin family) could play the main role in the infection process. Since AprX may be involved in the process of disease induction by degradation of host proteins that are present on cell surface and in mucus, modulation of host immune response and dissemination as well as its survival in host tissues and blood. Moreover, the AprX gene has toxic effect on cultured fish cells (**Zhang *et al.*, 2009)**.

The present study was planned to (1) isolate, identify and characterize *Ps. fluorescens* from *O. niloticus* in Kafr El-Sheikh governorate, Egypt, (2) investigate the seasonal incidence of *Ps. fluorescens* in *O. niloticus*, (3) screen the presence of one virulence-associated gene in the pathogenic *Ps. fluorescens* isolates that may be involved in the pathogencity of the organism, as well as (4) Application of antibiotic sensitivity test to be a helping tool in the control of Pseudomoniasis.

2. Materials and Methods

General layout of experiment

This study was carried out along the four seasons of the year 2016atthelaboratoryofFish Diseases and Management; andin accordance with recommendations of the Guide lines of Animal Care and Use of lab animals in the research at Faculty of Veterinary Medicine, Kafr El-Sheikh University, Egypt.

Fish samples:

A total number of 300 cultured *O. niloticus* fish were randomly collected from freshwater fish farmsat Kafr El-Sheikh governorate, Egypt. The collected fishes were transferred alive to the wet lab., Fish Diseases and Management Department, Faculty of Veterinary Medicine, Kafr El-Sheikh University, Egypt **(Langdon & Jones, 2002).**

Clinical examination:

The alive collected fishes were subjected to full clinical and post-mortem examination as described by ***(Schäperclaus 1992)***.

Isolation and identification of *Ps. fluorescens:*

For bacteriological examination, sterile swabs were aseptically collected from the infected parts, liver, kidney and spleen of collected naturally infected fishes. The collected swabs were firstly pre-enriched on Tryptic Soy Broth (TSB, Oxoid). After which (pre-enrichment on TSB) they were streaked on Pseudomonas-Fagar (Oxoid) for presumptive identification and incubated at 28 °C for 24-48 hours. Well-differentiated single bacterial colonies were further streaked onto Tryptic Soy Agar (TSA, Oxoid) for obtaining pure culture.

Biochemical analysis:

For phenotypic identification, pure cultures were subjected to Gram staining and viewed under the light microscope (Leica). Further biochemical tests like Pigmentation tests, motility, oxidase, catalase, H_2Sproduction, urease, triple sugar iron, indole production, methyl red, voges proskauer, citrate utilization, starch hydrolysis, gelatin liquefaction, oxidation fermentation test and sugar utilization tests, were performed for the identification as well as strain differentiation of bacteria **(Austin and Austin 2007);** and the obtained results were compared with the reference strain of *Ps. fluorescens* (obtained from the central lab., Faculty of Veterinary Medicine, Banha University, Egypt).

Molecular identification by PCR:

DNA extraction of *Ps. fluorescens* was performed by obtaining the pure culture then grown on nutrient broth and incubated at 37°C for 24 hours. Accurately, 4.2 ml of the bacterial cultures were harvested by centrifugation at 14,000 rpm. The cell pellet was suspended in 50 µl of TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0). Further, 300 µl of PCI reagent (phenol–chloroform–isoamyl alcohol) were added and mixed to release plasmid DNA. From this suspension, a 5 µl aliquot was directly used as a template for PCR amplification **(Ferrus *et al.*, 1999).**

Primer sequences used for PCR identification of *Ps. fluorescens:*

Application of PCR for identification of *Ps. fluorescens*by identification of virulence gene (aprX) extracellular alkaline metalloprotease of the isolated *Ps. fluorescens*was performed essentially by using Primers (Pharmacia Biotech) as shown in table 1.

DNA amplification for the selected virulent gene:

PCR amplification of extracellular alkaline protease (aprX) as virulence gene for *Ps. fluorescens*was carried out in a final reaction volume of 50 mL containing 20 ng of DNA, 0.2 mM of each dNTP, 1.25 U Taq DNA polymerase, PCR buffer and 0.2 mM of the primer. The PCR was applied under the following cycling conditions: initial denaturation at 94 C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 40 sec and 72°C for 1 min and a final extension step at 72°C for 7 min. A reaction mixture containing sterilized distilled water as the template was used as a negative control. Amplified DNA fragments were analyzed by 2% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBEbuffer stained with ethidium bromide and captured as well as visualized on UV transilluminator **(Machado *et al.*, 2013).**

Antibiotic sensitivity studies of the isolated *Ps. fluorescens:*

Ps. Fluorescens isolated strain were tested for antibiotic sensitivity test by the single diffusion

method according to **Panta *et al.* (2013)**. Sensitivity discs with variable concentrations were used to determine the susceptibility of the isolated bacteria **(***Oxoid Limited, Basingstoke, Hampshire, UK***).**

Agar plate method was applied by using of nutrient agar as a substrate for growth of the tested bacterium for its antibiotic sensitivity. The bacterial culture was uniformly spread on the surface of nutrient agar. Then the antibiotic discs were placed over the surface of inoculated plate. Moreover, the plate was then incubated at suitable temperature (25°C) for 2-7 days and checked for the growth of the bacterium around the antibiotic discs. The maximal inhibition zone for the growth of microbe is said to that antibiotic had maximum effect on the microbe growth.

Therefore, the antimicrobial discs and their concentrations as well as the diameters of the zones of inhibition for the tested strains was applied according to the guidelines stipulated by **National Committee for Clinical Laboratory Standards "NCCLS" (2001)**.

The tested strains were evaluated as susceptible, intermediate and resistant.

Experimental infection:

Total Bacterial count:

The pour plate method for estimation of *Ps. Fluorescens* strains per one ml, was used in demonstration of the inoculum dose for the experimental studies according to **Cruickshank *et al.* (1975)**.

Lethal Dose fifty (LD_{50}):

A total number of 70apparently healthy *O. niloticus* fish weighting 40±0.5 gm, were divided into seven groups, (10 fish each) and the seventh group was kept as a control group. All fishes were kept for 15 days under observation prior to injection for accommodation and to confirm that they are free from diseases. 24 hours colony culture of *Ps. fluorescens*strain on TSA were used. The colonies were picked up and suspended in sterile saline in a tenfold serial dilution with subsequent incubation at 28°C for 24 hours for plate counts on TSA. Only the dilutions (10^2-10^7cfu) were used. Each group was intra-peritoneally injected with 0.5 ml/fish of each bacterial dilution. The fishes in the control group were injected with 0.5 ml PBS/fish. All the injected fish were kept for one week post-inoculation for observation. The mortalities were recorded twice/day according to **Mastan (2013).** The freshly dead fishes were moved for further post-mortem examination. The LD_{50} (the dose which kills 50% of the injected fish) was calculated according to **Reed and Muench (1938).**

Pathogenicity test:

Experimental infection was carried out to determine the pathogenicity of *Ps. Fluorescens* strain using intra-peritoneal route injection according to **Eissa *et al.* (2010).** A total number of 60apparently healthy *O. niloticus* fish weighting 40±0.5 gm, were divided into three groups, (20fishes each). Each fish in the 1stgroup was intra-peritoneally injected with 0.2 ml/fish of LD_{50} dose of *Ps. Fluorescens* strain which was determined previously (3 x 10^5cfu). Each fish in the 2nd group (control negative group), was intra-peritoneally injected with 0.2 ml/fish of PBS. Each fish in the 3rd group (Control positive group), was intra-peritoneally injected with 0.2 ml/fish of the obtained reference *Ps. Fluorescens* strain. All injected fishes were observed for a period of 28 days post-inoculation. Mortalities were recorded daily and freshly dead fishes were moved for further pm examination and histopathological studies.

Histopathological examination:

Specimens from liver and kidney of experimentally infected fishes were taken. Specimens were fixed immediately in 10% neutral buffered formalin, dehydrated and embedded in paraffin blocks. Paraffin blocks were sectioned at 4-5 µm thickness and stained with Hematoxylin and Eosin (H & E) and examined under light microscope (Leica) using ×200 and ×400 magnification power according to **Bancroft and Gamble (2007).**

Table 1. The primer used for the amplification of virulence associated gene of *Ps. Fluorescens* isolated from *O. niloticus*.

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
aprX (F)	**5′** TAYGGBTTCAAYTCCAAYAC **′3**	194	**Bach *et al.* (2001)**
aprX (R)	**5′** VGCGATSGAMACRTTRCC **′3**		

3. Results and discussion

External examination of naturally infected *O. niloticus* revealed cloudiness of eyes, congested gills, haemorrhagic skin lesions and at the base offins and scales detachment (Figure 1 and 2). However, the postmortem findings exhibited accumulation of ascitic fluid in the peritoneal cavity, congested gills and liver with petechial haemorrhages (Figure 3).

The lesions displayed in the current study are similar to that reported by **(Ilhan *et al.*, 2006; Hanna *et al.*, 2014 and Darak and Barde, 2015)**. The clinical symptoms and postmortem findings may be attributed to the action of extracellular toxins of *Ps. Fluorescens* **(Khalil *et al.*, 2010).**

In the present study, the presumptive identification of the *Ps. fluorescens* was carried out from the colony morphology over Pseudomonas- F agar medium which acts as a selective medium for *Ps. fluorescens*. They produced circular, convex, smooth colonies, 2-3 mm in diameter, orange in colour and produce faint greenish coloration after 72 hours. While, on Pseudomonas-F agar, it appeared as yellowish green colonies, 2-3 mm in diameter and produce fluorescence after 48 hrs of incubation. This obtained result is similar to that obtained by **Masbouba, (2004)** & **Darak and Barde (2015)**.

The bacteria were observed as Gram-negative motile rods by polar flagella microscopically**;** the result which coincides with those reported by **Lopez *et al.* (2012) and Mastan, (2013.** Biochemical characterizations have proven to be a valuable method for typing and differentiation of bacterial fish pathogens (**Darak and Barde, 2015 and Lopez *et al.*, 2012)**.

In the present study, based on 18 biochemical tests, a total number of 51/300 isolates were positively identified as *Ps. fluorescens*. All strains were positive for Gram staining, pigmentation test, motility, oxidase, catalase, H_2S, urease, triple sugar iron, indole, methyl red, Voges Proskauer, citrate utilization, starch hydrolysis, gelatin liquefaction, oxidation-fermentation test and sugar utilization tests.

All strains were positive for pigmentation, motility, oxidase, catalase, urease, methyl red, citrate utilization, gelatin liquefaction, oxidation-fermentation test, sorbitol and sucrose utilization tests. However, they were negative for gram staining, H_2S, triple sugar iron, indole, Voges Proskauer, starch hydrolysis and glucose utilization test (Table 2).

Most of the phenotypic characteristics of the isolates were similar to those reported in Bergey's manual of determinative bacteriology **(Holt *et al.*, 1994 and Masbouba, 2004)**. Based on the biochemical tests results, all obtained isolates were similar to *Ps. fluorescens* reference strain except in urease test. The obtained biochemical results were similar to findings of **El-Moghazy, (2004); Masbouba, (2004); Mastan, (2013) and Darak and Barde, (2015**).

Regarding the seasonal prevalence of the isolated *Ps. fluorescens* strains within the seasons, it was revealed that the highest incidence was during the winter season (41.2%), autumn (25.5%), spring (19.6%) and summer (13.7%). On the other, *Ps. fluorescens* was positively recorded in 51 out of 300 *O. niloticus* examined cases with a percent 17%. This result coincided with **Masbouba, (2004); Swain *et al.*, (2007)** and **Mahfouz *et al.*, (2016)**. However, it is in contrast to **El-Galagel (2015).**

Table 2. Comparison of the phenotypic and biochemical characteristics between the isolated *Ps. Fluorescens* strains and the reference strain

Biochemical Tests	**Results**	
	Pseudomonas fluorescens **Strains**	
	Isolated strain	**Reference strain**
Gram staining	-	-
Pigment	+	+
Motility	+	+
Oxidase	+	+
Catalase	+	+
H_2S	-	-
Urease	+	-
Triple Sugar Iron	-	-
Indole	-	-
Methyl Red	+	+
VogusProskauer	-	-
Citrate utilization	+	+
Starch Hydrolysis	-	-
Gelatin Liquifaction	+	+
Oxidation/fermentation	+	+
Glucose	-	-
Sorbitol	+	+
Sucrose	+	+

+ = positive reaction,- = negative reaction

The PCR amplification of one *Ps. fluorescens* positive strain was screened for the presence of one virulence gene extracellular alkaline metalloprotease (aprX), by PCR technique. The results revealed that screened *Ps. fluorescens* strain had the virulence gene (aprX) (Figure 4).

Ps.fluorescens is an aquaculture pathogen that can infect a wide range of fish species. It has been reported that the virulence gene (*aprX*), obtained in the current study was specific to *Ps. fluorescens* (**Liu *et al.*, 2015**). The virulence of *Ps. fluorescens* may be related to the presence of extracellular alkaline metalloprotease (AprX) as it causes impaired abilities in the interaction with cultured host cells, makes adherence to host mucus, modulation of host immune response and dissemination and survival in host tissues and blood, also (AprX) has toxic effect on the cultured fish cells; so the (aprX) may be involved in the *Ps. fluorescens* virulence, (**Zhang *et al.*, 2009; Méndez *et al.*, 2012).**

The LD_{50} experiment in the present study revealed that the concentration 3×10^5cfu was the most potent dilution causing 50% mortalities within (24-48 hr). This result is similar to **Masbouba (2004) and Mastan (2013)** but in contrast to **Eissa *et al.*, (2010);** this may be attributed to the difference in fish species, ages and localities**.** The LD_{50} experiment with *Ps.*

fluorescens strain in *O. niloticus* revealed a higher mortality rate which may be due to the presence of virulence genes (**Zhang *et al.*, 2009; Méndez *et al.*, 2012).**

The clinical signs of experimentally infected *O. niloticus* with *Ps. fluorescens* strain were similar to natural infection in the form dark discoloration, fin rot, hemorrhage of the body, scale loss and congestion of the internal organs, these signs similar to that reported by **Miyazaki *et al.* (1984); Badran (1993) and Azza *et al.* (2002).** Hemorrhage of the body surface may be attributed to the aprX gene of *Ps. Fluorescens* which has toxic effect on fish cells (**Zhang *et al.*, 2009**).

Concerning the results of antibiotic sensitivity test of the isolated strains in the present study are shown in **Table (4).** The results revealed that *Ps. fluorescens* strain are highly sensitive to Amikacin (96.1%) and highly resistant to Ampicillin and Streptomycin (100%). This results may be attributed to that these strains has been exposed to un-proper dose and/or course, or uncontrolled use of antibiotics in the field by the farm owner in the treatment and physicochemical properties of the cell wall rather than the antibiotic inhibiting enzymes (**Koncicki and Szubstmaka, 1988; khalil *et al.*, 2010 and Abdel Tawab *et al.*, 2016).**

In the present study, the histopathological findings of the experimentally infected *O. niloticus* with *Ps. fluorescens* demonstrating congestion of the hepatic blood vessels associated with occasional thrombosis (Fig. 5), and necrotic foci could be observed (Fig. 6). The kidney showed severe congestion of the renal blood capillaries, interstitial haemorrhages and focal coagulative necrosis of renal tubules (Fig. 7). These findings were nearly similar to those reported by **Miyazaki *et al.* (1984**).

Table (3): Seasonal prevalence of *Pseudomonas fluorescens* among the examined *Oreochromis niloticus*:

Season	Total no. of examined fish	Positive infected cases within the season		% of isolated *Ps.fluorescens* from the total isolates
		No.	%	%
Winter	75	21	28%	41.2%
Spring	75	10	13.3%	19.6%
Summer	75	7	9.3%	13.7%
Autumn	75	13	17.3%	25.5%
Total	300	51	17%	100%

Table 4. Antimicrobial susceptibility of fifty one *Ps. fluorescens***strain**

Antimicrobial agents	Susceptible		Intermediate		Resistant	
	No.	**%**	**No.**	%	No.	**%**
Ampicillin (AM)	-	-	-	-	51	100
Streptomycin (S)	-	-	-	-	51	100
Erythromycin (E)	-	-	2	3.9	49	96.1
Penicillin (P)	-	-	3	5.9	48	94.1
Chloramphenicol (C)	34	66.7	3	5.9	12	23.5
Nalidixic acid (NA)	35	68.6	4	7.8	10	19.6
Sulphamethoxazol (SXT)	38	74.5	1	1.9	10	19.6
Neomycin (N)	39	76.5	3	5.9	9	17.6
Oxytetracycline (T)	41	80.4	2	3.9	8	15.7
Ciprofloxacin (CP)	42	82.4	1	1.9	8	15.7
Gentamicin (G)	44	86.3	2	3.9	5	9.8
Netilmicin (Net)	45	88.2	2	3.9	4	7.8
Kanamycin (K)	46	90.2	3	5.9	2	3.9
Amikacin (AK)	49	96.1	1	1.9	1	1.9

Figure 1. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *Ps. fluorescens* showing tail rot (blue arrow).

Figure 2. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *Ps. fluorescens* showing scale detachement (blue arrow).

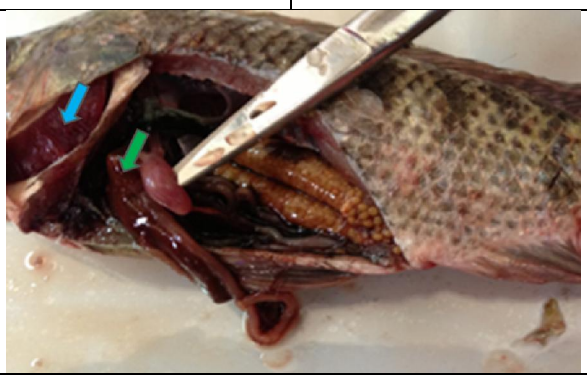

Figure 3. *Oreochromis niloticus*, collected from Kafr El-Sheikh farm, Egypt in summer, naturally infected with *Ps. fluorescens* showing congested gills (blue arrow) and liver (green arrows).

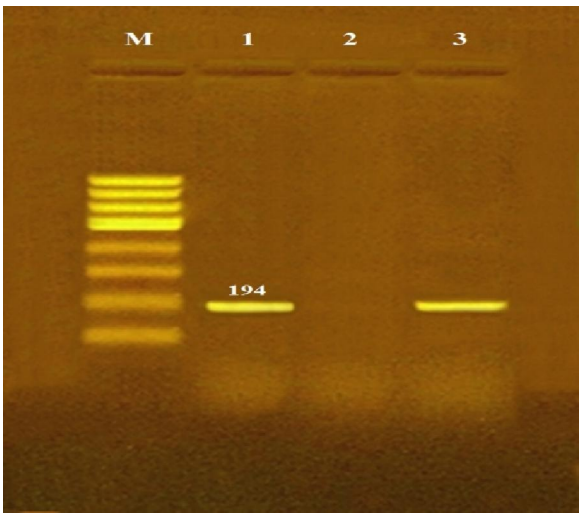

Figure 4. Agarose gel electrophoresis of PCR of aprX (194 bp) as virulent gene for characterization of *Ps.fluorescens*
Lane M: 100 bp ladder as molecular size DNA marker.
Lane 1: Control positive for aprX gene.
Lane 2: Control negative. Lane 3: Positive *Ps. fluorescens* for aprX gene.

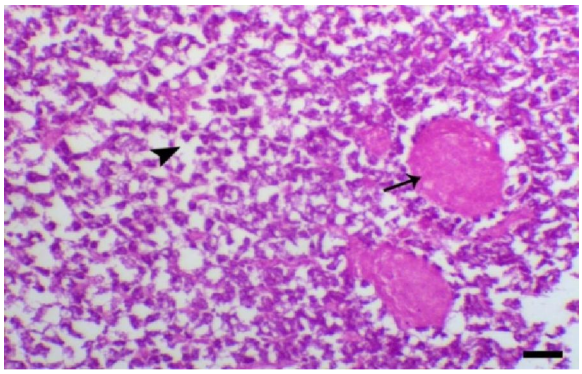

Figure 5: Liver of *O. niloticus* fish infected with *Ps. Fluorescens* showing hepatocytes vacuolation (arrowhead) associated with vascular thrombosis (arrow), H & E, X200.

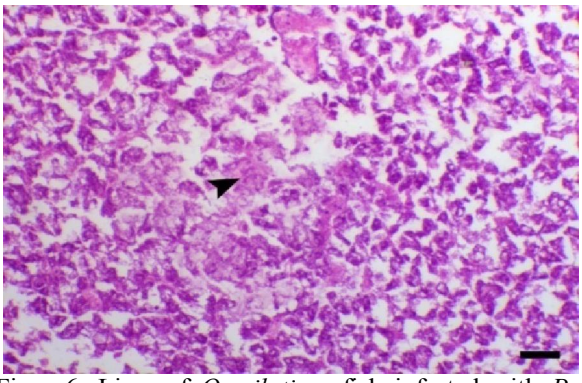

Figure6: Liver of *O. niloticus* fish infected with *Ps. fluorescens*showing necrotic foci (arrowhead), H & E, X200.

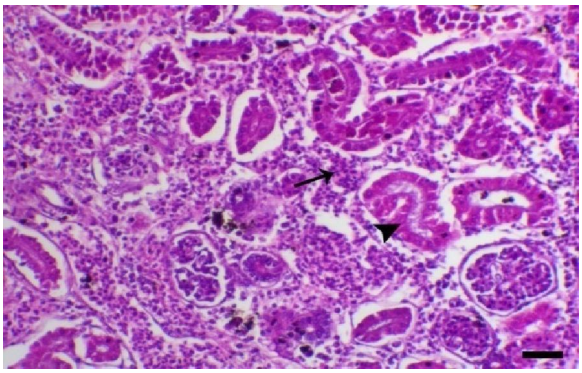

Figure7: Kidney of *O. niloticus*fish infected with *Ps. fluorescens*showing interstitial haemorrhages (arrow), leukocytic infiltration and degeneration of renal tubules (arrowhead), H & E, X200.

Conclusion:

Pseudomonasfluorescens; the ubiquitous facultative microorganism, is a potential pathogen having a serious threat to freshwater aquaculture and fish industry. Thesymptoms appeared as haemorrhagic lesions on the skinand fins, scales detachment, accumulation of ascitic fluid in the peritoneal cavity and congested gills and liver with petechial haemorrhages. The seasonal prevalence of 51 isolated *Ps. fluorescens*strains from 300 examined *O. niloticus* indicated that the highest incidence was during the winter season (41.2%), autumn (25.5%), spring (19.6%) and summer (13.7%), respectively. The PCR results revealed the presence of the aprX virulence-associated gene in the examined strain which may be responsible for the pathgencity of *Ps. fluorescens.* The antibiogram revealed that *Ps. fluorescens* strains are highly sensitive to Amikacin (96.1%) and highly resistant to Ampicillin and Streptomycin (100%).

Competing interests

Authors have declared that there is no competing interest.

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