Studies on Pseudomonas Septicemia in Some Tilapia in Ismailia

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Abstract

A total of 200 fish, 100 *Oreochromis niloticus* weighing (60-300 g) and 100 *Tilapia zillii* weighing (40-70 g) were collected randomly from different sites in Ismailia Governorate Egypt, during the period from October 2015 to September 2016. The clinical signs and postmortem lesions in naturally infected fishes were represented as hemorrhages on external body surface, hemorrhagic ulcer and congestion in most internal organs. The total prevalence of isolated *Ps. aeruginosa* in *O. niloticus* was 40% with the highest prevalence in winter 60% while the lowest prevalence was in summer 24%. The highest intensity was from liver 75% while the lowest was from gills 20%. On the other hand, prevalence in *T. zillii* was 22% with the highest in winter 36% while the lowest prevalence was in summer 8%. The highest intensity was from liver 72.7% while the lowest was from gills 13.6%. PCR is sensitive, rapid and specific method to detect resistant genes (*MexA* at 293 bp and *MexB* at 244 bp) and 16S rRNA gene at 530 bp in the selected isolates of *Ps. aeruginosa*. The antibiogramme of *Ps. aeruginosa* isolates showed high sensitivity to ampicillin, cefalexin and trimethoprim/sulfamethoxazole while they were resistant to amoxicillin and tobramycin. Challenge test revealed that the mortality rate in *O. niloticus and T. zillii* with *Ps. aeruginosa* by I/P route representing 80 and 50% of the total fishes, respectively. It was concluded that, *O. niloticus* is more susceptible to Pseudomonas septicemia than *T. zillii* in both natural and experimental infection.

Introduction

Fish has become an important resource in Egypt to meet the food and nutrition security needs of a rapidly expanding human population. Tilapia were mild, white flesh that is appealing to consumers, easy to rise and harvest, making them a good aquaculture species ***(Khalil et al., 2010)***.

Bacterial fish diseases were the major problems in aquaculture as it found naturally in the fish environment and under certain stress conditions caused severe economic losses ***(Olsson et al., 1998)***. Pseudomonades were

opportunistic Gram negative pathogens, causes outbreak when the normal environmental conditions changed **(*Roberts*, 1989)**. *Ps. fluorescens*, *Ps. angulliseptica*, *Ps. aeruginosa* and *Ps. putida* were identified in various species of fish as causative agents of pseudomonas septicemia ***(El-Nagar, 2010)***.

Among DNA marker, the Polymerase Chain Reaction (PCR) was highly sensitive specific and rapid method which improved the detection of *Ps. aeruginosa*e specially when using species-specific primer ***(Xu et al., 2004).***

Therefore, the aim of this work was to determine the clinical signs and postmortum lesions in naturally and experimentally infected Tilapias in addition to, isolation and identification of the causative agents of pseudomonas septicemia. Besides, studying total and seasonal prevalence, intensity in different organs of the infected fishes, the antibiogramme of the isolated strain and polymerase Chain Reaction (PCR) for confirmation of identification and for detection of antibiotic resistant genes (*mexA* and *mexB*).

Material and Methods

Collection of Fish Sample: A total of 200 fish, 100 *O. niloticus* (60-300 g) and 100 *T. zillii* (40-70 g) were collected randomly from different sites in Ismailia Governorate Egypt, during the period from October, 2015 to September, 2016. Live fish samples were placed in plastic bags and brought to the Animal Health Research Institute, Ismailia branch under standard measures of transportation. Upon arrival, the fishes were subjected to clinical and bacteriological examination ***(Plumb and Bowser, 1982)***, observed signs and detected lesions were recorded. Then, they were subjected to bacteriological examination.

Bacterial isolation and identification: Specimens from gills, liver, kidneys, intestine and spleen were collected under complete aseptic conditions. They were cultured directly onto plates of Pseudomonas isolation agar ***(Buller, 2004)***, 5 % sheep blood agar and nutrient agar. The plates were incubated at 37 ˚C for 24-48 hrs. After the recommended incubation period for each type of media; each type colony was picked up and re-streaked on a new plate of its original culture media and re-incubated at the same temperature and period. When the pure colonies were grown; a loopful of each pure culture was inoculated into a nutrient slope agar as a stock culture for biochemical identification of isolates by Vitek2 compact system (bioMe'rieux, Marcy 1' Etoile, France) ***(Barry et al., 2003)*** and for preservation of the microorganism.

Pathogensity Test: A total number of 40 apparently healthy fish, (20) *O. niloticus* weighing (70 ± 5 g) and (20) *T. zillii* weighing *(40* ± 5 g*)* were reared in well prepared

aquaria. They were allowed to acclimate to lab conditions for one week at 25 ± 1°C and fed with commercial pelleted ration at 3% of bodyweight. They were divided in to two groups A (treated group n=10) were injected intraperitonially with dose 0.2 ml of 24 hrs trypticase soya broth culture (3×10^7 living bacterial cell \ ml) according to ***Reed and Muench (1938)*** after matching with McFarland tube and B (control group n=10) were injected intraperitonially with dose 0.2 ml of sterile trypticase soya broth. Injected fishes were reared for 14 days and mortalities and clinical signs were recorded.

Molecular typing: The procedures of DNA extraction were carried out according to the methods described by ***Touihri et al., (2009)***. Oligonucleatide primers used for detection of *P. aeruginosa* have specific sequence and amplify a specific product as shown in table (1).

Table (1): Oligonucleotide primers sequences:-

Primer		Sequence	Product size	Reference
16S rRNA	F	ATGGAAATGCTGAAATTCGGC	530 bp	(Pirnay et al., 2000)
	R	CTTCTTCAGCTCGACGCGACG		
MexA	F	CGA CCA GGC CGT GAG CAA GCA GC	293 bp	(Xavier et al., 2010)
	R	GGA GAC CTTCGCCGC GTT GTC GC		
MexB	F	GTGTTCG-GCTCGCAGTACTC	244 bp	(Xavier et al., 2010)
	R	AACCGTCGGGATTGACCTTG		

Results

Clinical picture: The clinical signs and postmortem lesions in naturally infected fishes showed hemorrhages on external body surface, hemorrhagic ulcers, ascitis, detached tail and congestion in all internal organs with septicemic fluid in the abdomen (Plate 1, 2).

Bacteriological examination: The isolated bacteria were positive for oxidase, catalase, urease, citrate and gelatin liquefaction. While, they were negative for indole, V.P., methyl red and triple sugar iron agar (TSI). It hadn't the ability to produce acid from mannitol, glucose, sorbitol and sucrose. PCR is sensitive, rapid and specific method to detect resistant genes *(MexA* at 293 bp & *MexB* at 244 bp) and 16S rRNA gene at 530 bp in the selected isolates of *Ps. aeruginosa* figure (1, 2).

Total and seasonal prevalence: The total prevalence of *Ps. aeruginosa* in *O. niloticus* was 40% with the highest prevalence in winter 60% while the lowest prevalence was in summer 24%.

The highest intensity was from liver 75% while the lowest was from gills 20%. The total prevalence of *Ps. aeruginosa* in *T. zillii* was 22% with the highest prevalence in winter 36% while the lowest prevalence was in summer 8%. The highest intensity was from liver 72.7% while the lowest was from gills 13.6%.

Antibiogramme test: *Ps. aeruginosa* isolates were sensitive to ampicillin, cefalexin, cefpodoxime, ceftiofur, cefpirome, imipenem, amikacin, gentamicin and trimethoprim/sulfamethoxazole. In addition to, they were intermediate resistant to enrofloxacin, marbofloxacin and tetracycline while, they were resistant to amoxicillin, pipracillin, tobramycin and nitrofurantion.

Experimental infection: The challenge test revealed that the mortality rate in experimentally infected fishes (*O. niloticus and T. zillii*) with *Ps. aeruginosa* by I/P route representing 80% and 50% of the total fishes, respectively.

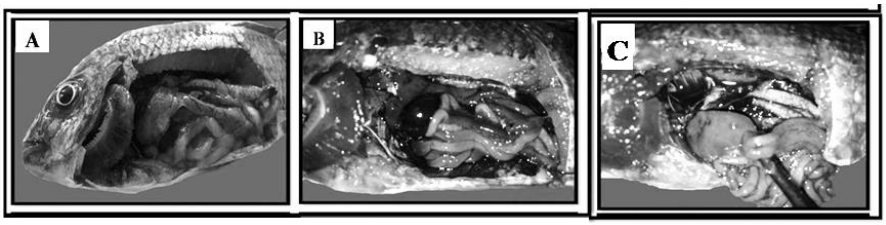

Plate (1): Clinical signs of infected fishes (*O. niloticus and T. zilii*)
A: *O. niloticus* showing hemorrhagic ulcer. **B:** *T. zilii* showing skin darkening, hemorrhage on gill cove, abdomen and tail erosion.

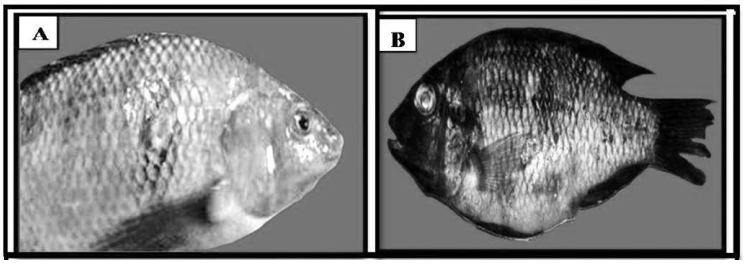

Plate (2): Post mortem lesions of infected fishes (*O. niloticus and T. zilii*)
A: *O. niloticus* showing congested liver and gill erosion. **B:** *O. niloticus* showing enlarged gallbladder. **C:** *O. niloticus* showing septicemic fluid in the abdomen.

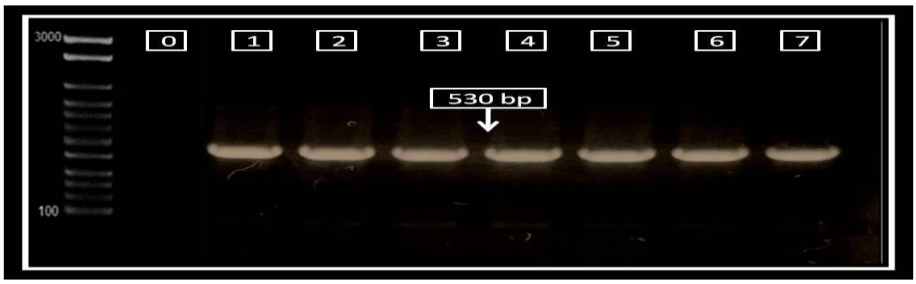

Figure (1): Detection of *Pseudomonas aeruginosa* 16s rRNA (530bp) gene by PCR. Lanes 0: negative control; lanes 1-7: *Pseudomonas aeruginosa* showing bands at 530bp.

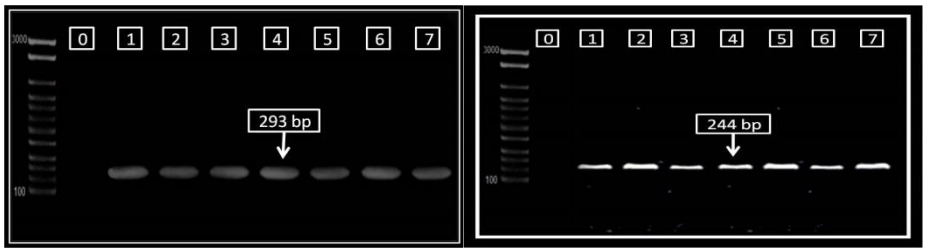

Figure (2): Detection of *Pseudomonas aeruginosa* resistant genes *MexA* (293bp) and *MexB* (244bp) by PCR. Lanes 0: negative control; lanes 1-7: *Pseudomonas aeruginosa* showing *MexA* and *MexB* bands at 293bp and 244bp respectively.

Discussion

The most observed clinical signs of infected tilapia were large irregular hemorrhages on body surface, in addition to exophthalmia, eye cloudiness and scales detachment, darkening of the skin, congested gills, ulceration of the skin and abdominal distention (ascites due to serohemorrhagic fluids). These results agreed with ***(Altinok et al., 2006; Austin and Austin, 2007; Eissa et al., 2010; Janga et al., 2014; Enany et al., 2016)***. These sings may be attributed to the action of the extracellular enzymes and degrading toxins ***(Todar, 2010)***.

The post mortem examination were varied lesions among the affected fishes as the liver was pale and congested in some fishes and congested with necrotic patches in other fishes, spleen and kidney were congested and enlarged in addition to the intestine was hyperemic and contained yellow mucous. Some other fishes showed signs of septicemia in all internal organs. These results in agreement with ***(Blanco et al., 2002; Altinok et al., 2007; Saleh and Azza, 2012; Enany et al., 2016)***.

Selective amplification of Pseudomonas 16S rRNA gene by PCR has been used to detect

differentiate Pseudomonas species**.** It was also used for genus or species level identification of *Ps. aeruginosa* ***(Drancourtn et al., 2000; Porteous et al., 2002).***

MexA and *MexB* resitant genes were found in *Ps. aeruginosa* by using specific primer designed by ***(Xavier et al., 2010).*** The electrophoresis of *MexA* and *MexB* genes PCR product was noticed with specific bands at 293 and 244 base pair respectively in agreement with ***(Lister et al., 2009)*** who studied *MexAB-OprM* (coding
gene: *MexA, MexB* and *OprM*) in *P. aeruginosa* which were genes for efflux pumps responsible for multidrug resistance as it pump antibiotics out of the cell.

The total prevalence of *Ps. aeruginosa* in naturally infected *O. niloticus* and *T. zillii* was 40% and 22% respectively. The obtained result of *T. zillii was similar to* ***El-Nagar (2010)***. But, lower prevalence was recorded by ***Masbouba (2004)*** who demonstrated that the prevalence of infection by *Ps. aureginosa* was (29.1%). In addition to ***Eissa et al. (2010)*** who concluded that *ps septicemia* was found in 30.83% of the 480 examined *O. niloticus* in fish farms in Egypt. On the other side, lower observation of *O. niloticus* infection by pseudomonas species was recorded by ***Zorrilla et al. (2003)*** who detected that low infection rates of pseudomonas among the examined marine fish (15.27 %). These differences may be due to fish species, age, nature of water, time and place of research.

The highest prevalence *of Ps. aeruginosa* infection in examined *O. niloticus* and *T. zilli* in different seasons was in winter (60%, 36%) followed by autumn (44%, 28%) then spring (32%, 16%) while the lowest prevalence was in summer (24%, 8%) respectively. These findings were in agreement with ***(El-Sayyad et al., 2010; Mastan, 2013)*** *and* ***Toranzo et al. (2005)***. While, disagreed with ***Eissa et al. (2011)*** who found that Pseudomonas species prevalence of infection was 43.33% (April 2008), 24.44% (August 2008), 21.11% (November 2008) and 17.77% (January 2009). These findings may be attributed to stress, including a lowered water temperature, may trigger outbreaks of pseudomonas septicemia ***Markovic et al. (1996)*** and ***Azza et al. (2002)***. The differences between the two types of fish in seasonal prevalence may be explained by the resistance of marine fish (*T. zilli*) to infection due to high water salinity.

Ps. aeruginosa were isolated from *O. niloticus* and *T. zilli* organs (liver, kidneys, spleen, intestine and gills) and the highest intensity was from liver followed by kidneys, spleen, intestine and the lowest was from gills. These results might be due to the organ most associated with the detoxification and biotransformation process is the liver and due its function, position and blood supply, it is also one of

the organs most affected by contaminants in the water ***(Camargo and Martinez, 2007)***. These findings agreed with ***(Eissa et al., 2010; El-Nagar, 2010).***

Ps. aeruginosa isolates were sensitive to ampicillin, cefalexin and trimethoprim/sulfamethoxazole while they were resistant to amoxicillin and tobramycin. These results in agreement with ***(Eissa et al., 2010; Khalil et al., 2010; López et al., 2012b; Abdullahi et al., 2013; Hanna et al., 2014)***. On the other hand, these results disagreed with ***Mastan (2013)*** who mentioned that amikacin did not show any effect against *Ps. aeruginosa* isolates. This might be explained by *Ps. aeruginosa* was able to develop mutational resistance and characterized by the biofilm mode of growth, which protects bacteria against antibiotics and the innate and adoptive defense mechanism ***(Fux et al., 2005)***.

The mortality rates in experimentally infected *O. niloticus* and *T. zilli* with *Ps. aeruginosa* by intra-peritoneal route were (80% and 50%) respectively. These results agreed with ***Enany et al. (2016)***. While, this findings disagreed with ***Austin and Stobie (1992)*** who stated that pseudomonads cause 100% mortalities within 7 days by I.P. or I.M. routes into rainbow trout. In addition to, ***Hossain et al. (2006)*** who reported that *Ps. aeruginosa* produced 30% mortality in *O. niloticus*. Besides, ***Hossian and Rahman (2011)*** who stated that Pseudomonas species cause 50% mortality in the experimental fishes. This may be attributed to differences in fish species, age and virulence of strain.

In conclusion, *O. niloticus* is more susceptible to Pseudomonas septicemia than *T. zillii* in both natural and experimental infection.

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الملخص العربى

أجريت هذه الدراسة علي عدد 200 سمكه من نوعين مختلفين من الاسماك ممثلين كالتالي: 100 سمكه من أسماك البلطي النيلي و 100 سمكه من أسماك الشبار الاخضر تم تجميعهم عشوائياً وموسمياً وفحصهما مباشرة من أماكن مختلفة من محافظة الاسماعيلية إبتداءاً من شهر أكتوبر 2015 وحتي شهر سبتمبر 2016. وذلك لعزل وتصنيف المسبب المرضي لمرض التسمم الدموي السودوموناسي في اسماك البلطي النيلي والشبار الاخضر خلال المواسم المختلفه عن طريق استخدام الطرق التقليدية و التقنيات الحديثة مثل (الايه بي اي 20 - جهاز الفيتك 2 و تفاعل البوليميريز المتسلسل). بالاضافة الي دراسة مدي حساسية الميكروب المعزول للمضادات الحيوية.

وقد أوضحت النتائج مايلي:

1- أهم العلامات الاكلينيكية الملحوظه في الاسماك المصابة (البلطي النيلي و الشبار الاخضر) هي: بقع نزفيه علي الجسم ، جحوظ و غيوم في العين، بروز فتحة الاخراج، تساقط للقشور، تغير لون الجلد واستسقاء في البطن.

2- أهم العلامات التشريحية للاسماك المصابة تباينت بين شحوب في اللون، احتقان و زيادة في الحجم للكبد- الطحال - الكلي – الامعاء والحوصله المرارية مع وجود سوائل بتجويف البطن.

3- كانت نسبة الاصابة الكلية بالسودوموناس ايريجينوزا في أسماك البلطي النيلي 40% بحيث كانت أعلي نسبة إصابة في موسم الشتاء (60%) بينما سجل موسم الصيف اقل نسبة اصابة (24%). كما سجل الكبد أعلي نسبة إصابة (75%) بينما سجلت الخياشيم اقل نسبة اصابة (20%).

4- كانت نسبة الاصابة الكلية بالسودوموناس ايريجينوزا في أسماك الشبار الاخضر 22% بحيث كانت أعلي نسبة إصابة في موسم الشتاء (36%) بينما سجل موسم الصيف اقل نسبة اصابة (8%). كما سجل الكبد أعلي نسبة إصابة (72.7%) بينما سجلت الخياشيم أقل نسبة إصابة (13.6%).

5- أوضح اختبار الحساسيه للسودوموناس ايريجينوزا انها حساسه للامبيسيلين و سيفاليكسين والترايميثوبريم/ السلفاميثوكسالين. بينما انه مقاوم للاموكسيسيلين والتوبراميسن.

6- أوضح اختبار العدوي علامات مرضية تشبة العلامات المرضية في الاسماك المصابة طبيعياً. وكانت نسبة النفوق في الاسماك (البلطي النيلي و الشبار الاخضر) المصابة بالعدوي المعملية بواسطة الحقن البريتوني 80 و 50%علي التوالي من مجموع الاسماك المعرضة للعدوي.