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Characterization and Pathogen Challenge of Pseudomonas Species from *Oreochromis niloticus* in Egypt.

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ABSTRACT

Fish diseases caused by Pseudomonas species are responsible for considerable economic losses in the commercial aquaculture of *Oreochromis niloticus* in Egypt. The present study was undertaken in order to draw up the characterization of pseudomonas species isolated from different localities in Egypt. The prevalence of Pseudomonas spp. isolated were 30 % from naturally infected *Oreochromis niloticus*, with highest prevalence of *P. fluorescence* (60%). All isolates were characterized by proteolytic, lipolytic activities and lecithinases production. Using16S-23S PCR ribotyping, the *P. fluorescens* isolates were divided for 4 biotypes. Fish challenge results showed that all *P. fluorescens* were pathogenic, while the *P. anguilliseptica* isolates were non-pathogenic. Most isolates were resistant to different antibiotics including cefotaxime.

Keywords: Pseudomonas species, pathogenicity, PCR-ribotyping,

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INTRODUCTION

Aquaculture is an expanding industry that needs to boost its global production and efficiency to meet the increasing consumer needs for fish. In Egypt, fish production comprises 20% of the white animal protein production, 17% of which is derived from aquaculture and the common cultured fish is *Tilapia nilotica* (*Oreochromis niloticus*) which have attained a great economic importance (Khalil *et al.,* 2010).

Bacterial diseases were considered the main cause of high mortalities and economic losses among fish. The genus Pseudomonas is a group of ubiquitous Gram negative pathogens; its members are saprophytic as well as pathogenic to plants, animals and humans. The most important fluorescent species are *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas chlororaphis* and plant pathogenic species (*Pseudomonas syringae* and *Pseudomonas chichorii*) (Roberts, 1989; Scarpellini *et al.,* 2004).

Pseudomonas fluorescens is an aquaculture pathogen that can infect many fish species, including Indian major carps, black carp, common carp, and Japanese flounder. Infection of fish by *P. fluorescens* leads to the development of the so-called Red Skin Disease, which can occur all year round and especially in fish injured by, for example, inappropriate handling and transportation. When the normal environmental conditions changed, the disease often leads to mortality, thus causing heavy economic losses (Swain *et al.,* 2007). Pseudomonas considers one of the most pathogenic bacteria affecting fish farms especially *O. niloticus* and *O. mossambicus* in Egypt (Khalil *et al.,* 2010).

MATERIAL AND METHODS

Microbial strains and phenotypic characterization

Random fish samples of *Tilapia nilotica* were caught from Al-Abbasa governmental fish farms, Kafr-Elsheikh private farm, and Al-Fayoum private fish farms respectively. Fishes were clinically examined for postmortem lesions. Samples from fish kidney and liver were inoculated onto Tryptic Soy Broth (Oxoid,UK) at 28ºC for 48 hrs. A loop was streaked onto MacConkey agar, Aeromonas agar and Pseudomonas agar medium plates then incubated at 28ºC for 48 hrs. Identification of all isolates was done by cultural, morphological and biochemical characters through using API-20E (Biomérieux). For long-term maintenance, stock solution cultures were stored in 20% (v/v) glycerol on Tryptic Soy Broth at -20^{o}C.

The production of extracellular proteolytic enzymes were determined on plate count agar (LABM, UK), supplemented with 1% skim milk powder. The appearance of a clear zone surrounding the colonies was interpreted as a positive proteolytic reaction. Also, production of extracellular lecithinase was determined by streaking of *P. fluorescens* isolates on PCA containing 10% egg yolk emulsion. The opaque rings surrounding the colonies were considered as a lecithinase-positive reaction after incubation at 7 and 26°C for 7 and 2 days, respectively (Dogan and Boor, 2003).

DNA extraction and PCR amplification

Template DNA was prepared by boiling 200 µL of bacterial suspension in distilled water in Eppendorf tubes for 10 min. The tubes were immediately cooled on ice and centrifuged (20,000 g, 1 min, 4°C); the supernatants were subsequently kept at -20°C. Three micro liters of template DNA suspension was used for each reaction. The PCR were performed in a volume of 50 µL containing 3 µL template DNA, 5 µL of 10 × PCR buffer, 200 µM of each dNTPs, 2 mM of MgCl2, 0.5 µM of each primer and 1.5 U of Taq Polymerase (Fermentas, Lithuania). The amplification was performed in a DNA thermal cycler (BioRad, Tgradient, USA).

DNA 16S region amplification for pseudomonas species was performed using the primer set 16SF–16SR (16SF 5´-AGAGTTTGATCCTGGCTCAG-3´; 16SR 5´- CTACGGCTACCTTGTTACGA-3´). Amplification of DNA 16S specific region of *P. fluorescens* was performed using the primer set 16SPSEfluF and 16SPSER (16SPSEfluF 5´-TGCATTCAAAACTGACTG-3´; 16SPSER 5´-AATCACACCGTGGTAACCG-3´) for amplification of a single DNA fragment of 850 bp (Scarpellini *et al.,* 2004). PCR-ribotyping of isolates was done using primers 23SR7 5´-GGTACTTAGATGTTTCAGTTC-3´; 16SR2 5´-TTGTACACACCGCCCGTCA-3´) according to Wang and Jayarao (2001).

Following amplification, 7 µL of the products were analyzed by electrophoresis at 100 V in 1% agarose gel (2% for ribotyping), containing 0.2 µg/mL of ethidium bromide using TAE buffer. The amplification was visualized in a Gel Documentation (Biometra, Germany).

The relatedness of the isolates was analyzed using PyElph software, version 1.3 (Pavel and Vasile, 2012). Results were obtained in phylogenetic tree based on clustering methods applied on the distance matrix, UPGMA (Unweighted Pair Group Method with Arithmetic Mean). A band position tolerance of 2.0% was used in analysis

Experimental infection

Apparently healthy *O. niloticus* fish, weighting 50 ± 5 gram were selected for determination of the pathogenicity of the bacterial isolates. Fishes were purchased from a commercial fish farm and maintained at 25°C in aerated dechlorinated water that was changed daily. Ten fishes were intra-peritonealy injected with selected bacterial cultures ($3x10^7$ CFU) of *P. fluorescens*, *P. anguilliseptica*, *P. putida* and *P. aeruginosa*. Fish were observed daily for 7 days. Daily mortality rate was recorded and dead, moribund and survivor fish were subjected to clinical and bacteriological examination.

Anti-biogram

The susceptibility of selected strains of *P. fluorescens, P. anguilliseptica, P. putida* and *P. aeruginosa* against different antimicrobial agents chloromphnicol, kanamycin, Gentamicin, clarithromycin Ampicillin, Amoxycillin, Nalidixic acid, metranidzole, Bactericin, Sulpha-Trimethobrim and Cefotaxime were investigated using the disc diffusion method according to CLSI guidelines (2007).

RESULTS AND DISCUSSION

The prevalence of *Pseudomonas* spp. isolated were 30 % (27/90) from naturally infected *Oreochromis niloticus,* whereas the percent of identified *P. fluorescence*, *P. putida, P. aeruginosa and P. anguilliseptica* species were 60, 10, 10, 10 and 10% respectively. The bacterial results indicated that the highest positive bacterial samples (60 %) were isolated from Al-Fayoum fish farms. The most prominent species was *P. fluorescens* followed by *P. aeruginosa*. Total of 20% of examined fishes from Kafr-ElSheikh farm were positive and species identified were *Pseudomonas anguilliseptica* and *P. Putida*. Only 10% of fishes from Al-Abbasa were identified as *P. fluorescens* (Table 1).

Table 1: Incidence of Pseudomonas species from different localities

Location	No. of Fish	Number and percentage	Bacterial species
Al Abbasa	30	3 (10%)	*P. fluorescens*
Kafr-Elsheikh	30	6 (20%)	*P. anguilliseptica (3), P. Putida (3)*
El-Fayoum	30	18 (60%)	*P. fluorescens (15), P. aeruginosa (3)*
Total	90	27 (30%)	

In aquaculture, *P. aeruginosa* and *P. fluorescens* especially are the most frequently isolated opportunistic pathogenic species causing hemorrhagic septicemia identical to that seen with Aeromonas septicemias. *P. fluorescens* affects freshwater and salt-water fish throughout the world. Infection of fish by *P. fluorescens* leads to the development of the so-called Red Skin Disease, and appear to be stress related disease of freshwater fish especially under culture conditions leading to severe economic losses (Zhang *et al.,* 2009). In addition, *P. anguilliseptica* causes red spot disease. It was first isolated from cultured Japanese eels *Anguilla japonica* affected by red spot disease (Wakabayasi & Egusa 1972). *P. anguilliseptica* then isolated from outbreaks in European eels in Scotland (Ellis *et al.,* 1983). Subsequently it has been isolated from different fish species in several countries.

This result is in accordance with the previous reports to identify the same organisms from Egypt. El-Hady and Samy (2011) isolated 55.3% of Pseudomonas spp. from 150 naturally infected *Oreochromis niloticus*

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in different localities of Behira, Dakahlya, Kalubya, Kafr el-Sheikh, Fayoum, Menofya, Sharkia. *P. fluorescence*, *P. putida, P. aeruginosa and P. anguilliseptica* species were identified in rates of 55.4, 20.5, 13.3 and 10.8% respectively.

In another experiment, the prevalence of Pseudomonas infections among the examined fish was 30.83% (148 from 480 fish). From them, 80 isolates were identified as *P. fluorescens Biovar I* (15 isolates), *P. fluorescens Biovar II* (17 isolates), *P. fluorescens Biovar III* (13 isolates), *P. anguilliseptica* (18 isolates), *P. putida* (5 isolates) and *P. aeruginosa* (12 isolates) (Eissa *et al.,* 2010).

Our results showed that all *P. fluorescens, P. anguilliseptica, P. putida* and *P. aeruginosa* isolates were observed to be proteolytic and lipolytic at 7 and 26°C. In contrast to our result, the previous studies showed that 80 and 91% of *P. fluorescens* isolates were proteolytic at 7 and 22°C, respectively while, only 7 and 44% of the isolates were lipolytic at the same temperatures. *P. fluorescens* was more likely to produce proteinases at 7 and 22°C and lipases at 22°C (Wang and Jayarao 2001). Many fluorescent Pseudomonads produce extracellular metalloproteases. Among the proteases that have been characterized on the genetic and biochemical level is AprX, which has been identified in several *P. fluorescens* strains. The AprX is an extracellular metalloprotease that is involved in bacterial virulence (Zhang *et al.* 2009).

The target regions for PCR primers 16SPSEfluF and 16SPSER were identified at locations 482–521 bp and 1311–1351 bp for all the investigated *P. fluorescens*. A single DNA fragment of 850 bp was amplified only for *P. fluorescens* (Fig. 1). The proposed PCR protocol (PSEfluF–PSER) offers a rapid diagnostic tool to identify a *P. fluorescens* member of Pseudomonas group 1 (Scarpellini *et al.,* 2004).

Phylogenetic analysis of some *P. fluorescens* was performed to estimate the genetic relatedness between *P. fluorescens* isolates using 16-23S primers set for PCR ribotyping (Fig. 2). As indicated from the result, the isolates of *P. fluorescens* were placed into 4 clusters. The isolated strains from Al-Fayoum fish farms were placed in three distinct clusters (F1, F2, F3), while Al-Abbasa isolates were placed in one cluster (B1). Wang and Jayarao (2001) used this method in genotypic characterization of *Pseudomonas* strains isolated from bulk tank milk and showed that the 16S-23S PCR ribotyping technique allowed differentiation of *P. fluorescens* isolates.

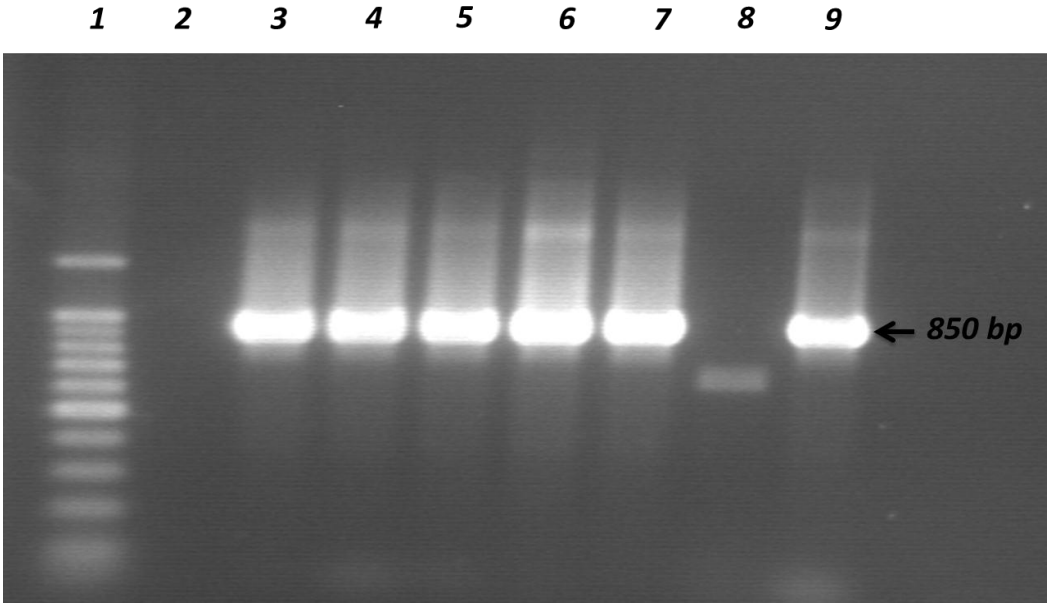

Figure 1: Agarose gel electrophoresis showing PCR of *P. fluorescens* isolates. Lane 1, 100 bp molecular marker. Lane 2, negative control. Lanes 3, 4, 5, 6, 7, and 9 are *P. fluorescens* isolates. Lane 8, *P. aeruginosa*.

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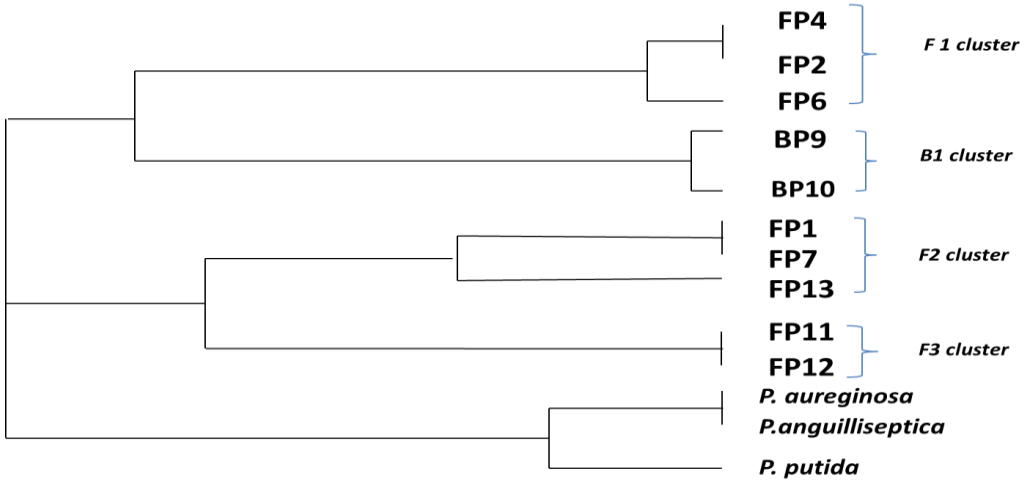

Figure 2: Phylogenetic tree of Pseudomonas isolates. Dendrogram showing the relationship between isolates of Pseudomonas species obtained After PCR-ribotyping.

To verify the pathogenicity of Pseudomonas isolates, selected strain from each species was injected into a group of fish. The results revealed that, groups challenged with *P. aeruginosa* showed no mortalities which proved that these strains were non pathogenic. While, those injected with *P. putida* and *P. aeruginosa* showed mortality rates of 20 and 60%, respectively by the fifth day. While, those injected with *P. fluorescens* strains showed variable mortality rates, the highest rate was for strain FP2 which caused death of all fishes within 24 hours, followed by strain FP6 (80%). Al-Abbasa strains were highly virulent and caused death for 80% of fishes within 72 hrs.

This result was in contrast to previous results done by Eissa *et al.* (2010) who reported that the isolated *P. fluorescens biovar I, II* and *III* were non-pathogenic. Also, he reported that the *P. anguilliseptica* was the highly pathogenic and induced 96.66% mortality. Also, our results were in contrary to the previous results published in Egypt which indicated that the *P. anguilliseptica* resulted in 100% mortality in *O. niloticus* (Sakr and Azza, 2008).

The challenged fish exhibited signs of dark pigmentation, easily detached scales, petechial hemorrhage on different parts of the body surface, and ulceration, especially at dorsum part and at the base of fins with eroded fins. Results of re-isolation of bacteria and its identification revealed the same characters of the injected bacteria.

The antibiogram sensitivity of *P. anguilliseptica, P. putida* and *P. aeruginosa* revealed that most of the isolates were sensitive to chloromphnicol, kanamycin, Gentamicin, clarithromycin. In addition, all species showed resistance to Ampicillin, Amoxycillin, Nalidixic acid, metranidzole, Bactericin and Sulpha-Trimethobrim. The *P. anguilliseptica and* All *P. fluorescens* but 2 isolates showed resistance to cefotaxime, while *P. putida* and *P. aeruginosa* isolates were intermediate resistant to cefotaxime.

CONCLUSION

Pseudomonas is one of the most pathogenic bacteria affecting fish farms in Egypt. Intra-peritoneally injected healthy fish showed that, all *P. fluorescens* isolates were pathogenic, while *P. anguilliseptica* were found to be a virulent. Our study showed that the 16S-23S PCR ribotyping technique allowed differentiation of *P. fluorescens* for 4 biotypes and PCR-ribotyping considers a rapid and accurate method for typing Pseudomonas.

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