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Molecular Typing of *Pseudomonas* Species Isolated from Some Cultured Fishes in Egypt

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Abstract: Samples from different species of cultured fishes were collected from different fish farms at different localities in Egypt for the isolation of *Pseudomonas* species. Four *Pseudomonas* species were recovered from naturally infected fishes; *Ps. fluorescence*, *Ps. putida, Ps. aeruginosa and Ps. anguilliseptica.* In-vitro sensitivity test of isolated *Pseudomonas* strains to different chemotherapeutic agents was conducted. Typing of the isolated *Pseudomonas* spp. was carried out by plasmid profile analysis as well as protein profile analysis by SDS-PAGE. It was clear from plasmid profile that most of the isolates showed a degree of variation in plasmid number (1 up to 4 plasmids) and molecular weight (494 up to 24279 bp), while, all *Pseudomonas* strains harbored a plasmid of 24279 bp. SDS-PAGE revealed that one isolate from *Pseudomonas fluores*cence, *Pseudomonas aeruginosa* and *Pseudomonas anguilliseptica* species shared in one band that was present at 144.8 kDa.

Key words: Ps. Fluorescence · Ps. Putida · Ps. Aeruginosa · Ps. Anguilliseptica · Plasmid Profile.

INTRODUCTION

Bacterial fish diseases constitute one of the major challenges facing sustainable aquaculture production. Most bacterial pathogens of fish are aerobic Gram-negative rods. Diagnosis is by isolating the organism in pure culture from infected tissues and identifying the bacterial agent [1]. Plasmid and protein profiling have provided the means for sub grouping many bacterial spp. and appear to be useful tools for characterizing strains from common sources. The term clone has been used to define apparently identical strains originating from a single source. Plasmid profiles have been found to be one of the best characteristics for the routine identification of bacteria originating from the same clone [2].

Recently, the Office International des Epizooties (OIE) (World Organization for Animal Health) has initiated the development of international recommendations on the detection and control of antimicrobial resistance as it relates to zoonotic bacteria and to resistance determinants that may be transferred between animals and from animals to humans. Surveillance information is necessary to determine the proportion of resistance to antimicrobials in defined populations, detect emerging resistance trends, provide a basis for policy recommendations and intervention within the animal and public health fields, assess the impact of interventions and provide information for prescribing practices and prudent use recommendations [3].

Pseudomonads exist throughout the aquatic environment and are associated with both healthy and diseased fish. It is generally believed that these bacteria can be opportunistic pathogens or produce damaging secondary infections [1]. *Pseudomonas fluorescence* is likely to be spread through water, which will serve as the primary reservoir of infection [4].

In Egypt, *Pseudomonas anguilliseptica* was isolated from naturally infected *Oreochromis niloticus* showing septicemic picture [5], also, Abd El-Ghany *et al*. [6] isolated *Pseudomonas aeruginosa* from *Oreochromis niloticus* with a percent of 50%. As well as *Pseudomonas putida* is isolated from diseased cultured *Oreochromis niloticus* [7]. *Pseudomonas fluorescence* has been isolated from gas bladder of diseased *Oreochromis niloticus* [8].

This study was done to investigate the existence of *Pseudomonas* spp. in some different types of cultured fishes in Egypt. It was also concerned about *in-vitro* sensitivity test of isolated *Pseudomonas* strains to different chemotherapeutic agents and typing of these isolates by plasmid profile analysis as well as protein profile analysis by SDS-PAGE.

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Table 1: Different localities of the examined cultured fishes.

Fish species	Location (Province)	No.
Oreochromis niloticus	Behira, Dakahlya, Kalubya, Kafr el-Sheikh, Fayoum, Menofya, Sharkia	150
Mugil cephalus	Behira, Kafr el-Sheikh	50
Cyprinus carpio	Kafr el-Sheikh, Sharkia	50
Hypophthalmichthys molitrix	Kafr el-Sheikh, Dakahlya	50

MATERIALS AND METHODS

Clinical and Postmortem Examination of Naturally Infected Fishes: A total number of 300 cultured fishes (150 *Oreochromis niloticus,* 50 *Cyprinus carpio*, 50 *Mugil cephalus* and 50 *Hypophthalmichthys molitrix*) was collected from different fish farms at different localities in Egypt (Table 1). Fish were examined clinically for any abnormal lesions according to Noga [9].

Isolation and Identification of *Pseudomonas* Spp. From Fishes: Samples from internal organs of examined fishes were streaked onto nutrient agar, trypticase soy agar, Rimler- Shotts medium (RS) and Pseudomonas agar medium plates then incubated at 28ºC for 24-48 hr. The growing colonies were picked up in pure form and identification of all isolates was done by cultural, morphological and biochemical characters according to Quinn *et al.* [10], Austin and Austin [4] and through using API-20E (Biomérieux) for Gram-negative fish pathogens.

***In-vitro* Antibiotic Sensitivity Test of Isolated *Pseudomonas* Spp:** Antibiotic susceptibility testing was performed by the disk diffusion method and interpreted in accordance with criteria of the National Committee for Clinical Laboratory Standards [11].

SDS Electrophoresis: Using low range molecular weight standard (Pharmacia) as marker (250, 148, 98, 64, 50, 36, 22kDa), electrophoresis was done using 10% separating gel and 4% stacking gel in denatured dissociating buffer system (SDS-PAGE) following the method of Laemmli [12] using Hoefer Scientific Instrument vertical apparatus.

Plasmid Profile Analysis: Bacterial strains were screened for plasmid DNA by the method described by Sambrook *et al.* [13] and Towner and Cockayne [14]. The Extracted plasmid DNA was electrophoresed on an 0.7% horizontal agarose gel containing 0.5ug of ethidium bromide solution per ml and analyzed under UV illumination.

RESULTS

Clinical and Post-mortem Examination of Naturally Infected Fishes: Naturally infected fishes showed hemorrhages all over the fish body especially at the base of fins, tail and fins rot; detachment of scales and skin ulceration and abdominal distention. Internally these fishes showed abdominal dropsy with reddish ascetic exudates; liver paleness and enlargement in some fishes and congestion with necrotic patches in other fishes; spleen was congested with enlarged and hemorrhagic enteritis in some fishes.

Isolation and Identification of *Pseudomonas* Spp. From Fishes: *Pseudomonas* spp. was isolated from *Oreochromis niloticus* with a percent of 55.3 and from *Mugil cephalus*, *Cyprinus carpio* and *Hypophthalmichthys molitrix* with 36, 44 and 40%, respectively (Table 2).

From naturally infected *Oreochromis niloticus* fish; *Ps. fluorescence*, *Ps. putida, Ps. aeruginosa and Ps. anguilliseptica* species were identified in rates of 55.4, 20.5, 13.3 and 10.8% respectively. From naturally infected *Mugil cephalus* only *Ps. fluorescence* was isolated with a rate of 100%. From naturally infected *Cyprinus carpio* fish; *Ps. fluorescence* and *Ps. aeruginosa* species were identified in rates of 54.5 and 45.5% respectively.

Table 2: Incidence of *Pseudomonas* spp. isolation from examined fishes.

		Positive isolation for *Pseudomonas* spp.	
Fish species	No. of examined fishes	NO.	%
Oreochromis niloticus	150	83	55.3
Mugil cephalus	50	18	36
Cyprinus carpio	50	22	44
Hypophthalmichthys molitrix	50	20	40
Total	300	143	47.7

Table 3: *Pseudomonas* spp. isolated from naturally infected fishes

			Identified spp.	
Fish species	+ve isolation	*Pseudomonas* spp.	No.	%
Oreochromis niloticus	83	*Ps. fluorescence*	46	55.4
		Ps. putida	17	20.5
		Ps. aeruginosa	11	13.3
		Ps. anguilliseptica	9	10.8
Mugil cephalus	18	*Ps. fluorescence*	18	100
Cyprinus carpio	22	*Ps. fluorescence*	12	54.5
		Ps. aeruginosa	10	45.5
Hypophthalmichthys molitrix	20	*Ps. fluorescence*	20	100

Table 4: *In-vitro* antibiotic sensitivity of *Pseudomonas* isolates.

		Isolates			
Chemotherapeutic agents	Concentration per disc	*Ps. fluorescence*	*Ps. putida*	*Ps.aeruginosa*	*Ps. anguilliseptica*
Amoxycillin	10 µg	R	R	R	R
Cephalothin	30 µg	R	R	R	R
Colistin sulphate	25 µg	S	S	S	R
Danofloxacin	5 µg	S	R	S	S
Erythromycin	15µg	R	R	R	R
Gentamycin	10 µg	S	S	I	S
Lincomycin	10 µg	R	R	R	R
Nalidixic acid	30 µg	S	R	S	I
Nitrofurantoin	300 µg	R	R	R	R
Oxolonic acid	2 µg	S	R	S	S
Oxytetracycline	30 µg	S	S	S	S
Sulphamethoxazole 23.75ug/ Trimethoprim 1.25 ug	25 µg	R	R	R	R

S: Sensitive I: Intermediate R: Resistant

From naturally infected *Hypophthalmichthys molitrix* fish only *Ps. fluorescence* was isolated with a rate of 100% as seen in table (3).

***In-vitro* Antibiotic Sensitivity Test of Isolated *Pseudomonas* Spp:** As shown in table (4) all *Pseudomonas* spp. isolates were sensitive to oxytetracycline 30 µg while all the examined isolates were resistant to amoxicillin 10µg, cephalothin 30µg, erythromycin 15µg, lincomycine 10µg, nitrofurantoin 300µg and sulphamethoxazole-trimethoprim 25 µg.

SDS Electrophoresis: All *Pseudomonas* strains electrophoretic protein profiles are shown in photo (1) which revealed that all the *Pseudomonas fluorescence* isolates showed common bands at 38.3, 41.4, 54.1, 84.9 and 132.1 kDa. *Pseudomonas putida* isolates shared in three bands which were present at 42.1, 62.6 and 121.3 kDa. *Pseudomonas aeruginosa* isolates shared in three bands which were present at 14.5, 39.5 and 47.9 kDa. *Pseudomonas anguilliseptica* isolates shared in three bands which were present at 14.6, 96.7 and 110.7 kDa. One isolate from *Pseudomonas fluorescence*, *Pseudomonas aeruginosa* and *Pseudomonas anguilliseptica* species shared in one band that was present at 144.8 kDa.

Plasmid Profile Analysis: Most of the isolates showed a degree of variation in plasmid number (1 up to 4 plasmids) and molecular weight (494 up to 24279 bp). As shown in photo (2) all *Pseudomonas* strains harbored a plasmid of 24279 bp, while all *Pseudomonas fluorescence* strains had plasmids of 24279, 7607, 4965 and 2300 bp. Meanwhile a plasmid of 494 bp was detected in one isolate of *Ps. Anguilleseptica*.

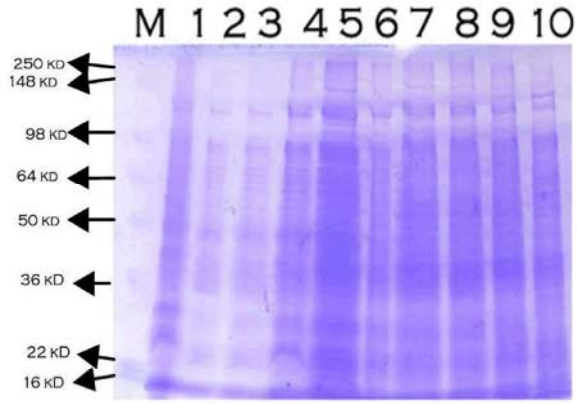

Photo 1: SDS of whole proteins of *Pseudomonas* species isolated from cultured fishes.
Lane M : marker. Lanes 1, 2, & 3: *Pseudomonas fluorescence*, Lanes 4 & 5: *Ps. putida*, Lanes 6& 7: *Ps. aeruginosa* and Lanes 8, 9, &10: *Ps. Anguilleseptica*

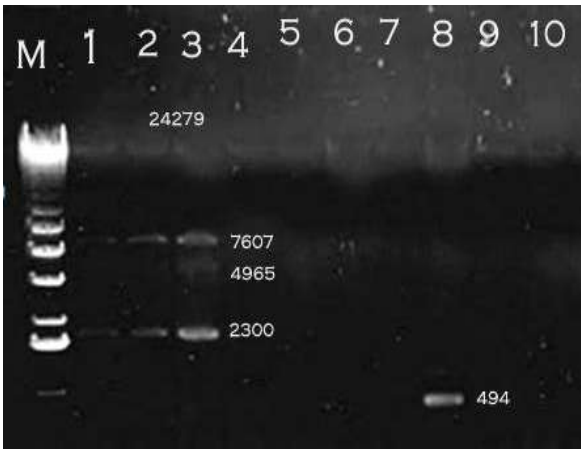

Photo 2: Plasmid profiles of *Pseudomonas* species isolated from cultured fishes.
Lane M : Molecular weight marker (12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 506, 396, 344, 298, 222, 201, 154, 134, bp.) Lanes 1, 2, & 3: *Pseudomonas fluorescence*, Lanes 4 & 5: *Ps. putida*, Lanes 6& 7: *Ps. aeruginosa* and Lanes 8, 9, &10: *Ps. Anguilleseptica*.

DISCUSSION

Our study revealed that naturally infected fishes with *Pseudomonas spp.* showed signs of infection which were reported in the results. Nearly similar findings were recorded by Marzouk *et al.* [15], Abd El-Ghany *et al*. [6] and Salama and Gharib [7].

In this study, the incidences of *Pseudomonas* spp. in the examined cultured fishes were 55.3% from *Oreochromis niloticus*, 36% from *Mugil cephalus*, 44% from *Cyprinus carpio* and 40% from *Hypophthalmichthys molitrix*. The prevalence of *Pseudomonas* spp. *Pseudomonas anguilliseptica* and *Pseudomonas putida* in cultured *Oreochromis niloticus* was reported as 25.5%, 63.5% and 12.5, respectively [5]. *Pseudomonas fluorescence* was isolated from 24% of diseased *Oreochromis niloticus* [8].

According to our work, all *Pseudomonas* spp. isolates were sensitive to oxytetracycline while all the examined isolates were resistant to amoxicillin, cephalothin, erythromycin, lincomycine, nitrofurantoin and sulphamethoxazole- trimethoprim and varied in sensitivity for the other examined antibacterial agents. These results confirmed previous finding by El-Hady and El-Katib [8] who reported that *Pseudomonas fluorescence* isolates are highly susceptible to oxytetracycline with different susceptibilities to the other tested chemotherapeutic agents. In this concern, *Pseudomonas fluorescence* isolate resistant to carbapenems was recovered during an environmental survey performed with water from the Seine River, Paris [16]. The antibiotic sensitivity profile of *Pseudomonas fluorescence* showed that it was resistant to amoxycillin, cloxacillin, penicillin-G and ampicillin, thus these antibiotics cannot be used as therapeutic agents for treatment [17].

Our results revealed that one isolate from *Pseudomonas fluorescence*, *Pseudomonas aeruginosa* and *Pseudomonas anguilliseptica* species shared in one band that was present at 144.8 kDa which may help in vaccine production against Pseudomonas and this need further study. On the other hand SDS- PAGE of extracellular products of *Pseudomonas fluorescence* isolated from freshwater fishes revealed the presence of one common band of about 35 kDa in all isolates [18].

From the point of our work all *Pseudomonas* strains were screened for plasmid DNA which revealed that most of the isolates showed a degree of variation in plasmid number (1 up to 4 plasmids) and molecular weight (494 up to 24279 bp). All *Pseudomonas* strains harbored a plasmid of 24279 bp, while all *pseudomonas fluorescence* strains had other plasmids of 7607, 4965 and 2300 bp. Meanwhile plasmid of 494 bp was detected in one isolate of *ps. Anguilleseptica*. In this concern, Negrete *et al.* [19] reported that all the plasmids extracted from the *Pseudomonas* strains isolated from ornamental fish presented ranges in common, between 3000 and 16363 bp, thus confirming that the bacteria received an antibiotic in

common. Ibrahim [20] reported that 6 *Pseudomonas aeruginosa* were isolated from ostriches and harbored one plasmid in each strain and their molecular weights were 33493, 28565, 24695, 28482 and 33882 bp.

The results obtained in this study showed that the electrophoretic profiles of *Pseudomonas spp*. isolated from all types of examined cultured fishes; *Pseudomonas fluorescence*, *Pseudomonas aeruginosa* and *Pseudomonas anguilliseptica* shared in one band that was present at 144.8 kDa. All *Pseudomonas* strains harbored a plasmid of 24279 bp.

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