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Molecular Detection of some Virulence Factors of *Pseudomonas aeruginosa* Isolated from Freshwater Fishes at Qalubiya Governorate, Egypt.

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ABSTRACT

The present study was conducted on 145 clinically diseased fish samples, 55 Nile tilapia*;* *Oreochromus niloticus* (*O.niloticus*); 65 African catfish (*C. gariepinus*), and 25 Grey mullet, *Mugilcephalus* (*M. cephalus*) of various sizes. Fishes were collected from different fish markets at Qalubiya Governorate, Egypt, during the period from September 2021 to May 2022 for isolation of *Pseudomonas (Ps) aeruginosa* with molecular studying of its virulence factors. The results cleared that 43 *Ps. aeruginosa* isolates (13.8%) were recovered as follow;14/55 from *O. niloticus* (25.4%), 19/65 from *C. gariepinus* (29.2%) and 10/25 from *M. cephalus* (40.0%). The isolated *Ps. aeruginosa* were highly resistant for ampicillin; penicillin-G followed by oxacillin; amoxicillin; cefotaxime and tetracycline. In contrast, they were sensitive to gentamicin followed by norfloxacin, ciprofloxacin and doxycycline. PCR results showed that, exotoxin A (*tox*A); outer membrane lipoprotein L (*opr*L) and exo polysaccharide synthesis locus (*psl*A) virulence gene were detected in three out of six studied *Ps. aeruginosa* isolates and exotoxin S (*exo*S) virulence gene was detected in five out of six studied ones. It is concluded that *Ps. Aeruginosa* has multiple antibiotic resistances and virulence genes, therefore it is an important pathogen causes Pseudomonas septicemia in fish and could have a public health concern for the consumers.

1. INTRODUCTION

Pseudomonas (*Ps*.) *aeruginosa* is considered as one of the most important fish pathogens. It is responsible for ulcer type diseases including ulcerative syndrome, Pseudomonas septicemia, which characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascites, and exophthalmia, leading to high mortalities and high economic losses (Hanna *et al*., 2014 and Abd El Tawab *et al*., 2016).

Ps. aeruginosa is a Gram-negative, motile, aerobic, non-spore forming bacillus. It characterized by its simple nutritional requirement, non-lactose fermenter on MacConkey agar, and secretion of a fluorescent yellow green siderophore under iron-limiting conditions (Markey *et al*., 2013; Shahrokhi *et al*., 2022).

Pseudomonas aeruginosa is considered problematic pathogens as they express cell associated virulence factors such as lipo-polysaccharide, alginate/biofilm, pili, and flagella. It also secrets extracellular products known as rhamnolipid, phospholipase C, hemolysin, lecithinase, protein exotoxin A, proteases and other exoenzymes; and all of them play a role in disease pathogenesis with severe and aggressive infections in humans, fish, and animals (Markey *et al*., 2013).

In addition, *Ps. Aeruginosa* has characterized by its resistance to wide range of commercial antibiotics. So, it seems to be more critical than other food-spoilage bacteria as it can transmit its multi-drug-resistance (MDR) plasmids to human-being after consumption of contaminated under-cooked fish and fish products with MDR *Ps. aeruginosa* (Shahrokhi *et al*., 2022). Moreover, the natural resistance of *Ps. aeruginosa* to several group of antibiotics, and the refractory to disinfectants together with the ability to biofilm formation make this bacterium blamed for production of virulent exopolysaccharides (EPSs) such as *psl*; which are playing a role in both cell to cell, and cell to substrate attachment leading to high morbidity and mortality rates within the infected fish population (Ghafoor et al., 2011; Yang *et al*., 2011). The *psl*A gene is mainly responsible for formation of biofilm (Nader *et al*., 2017 and Abdulhaq *et al*., 2020).

As *Ps. aeruginosa* is considered as one of the most vigorous zoonotic fish pathogens, with great economic importance in Egypt. Therefore, the present study was conducted to throw light over their infection in some freshwater fishes, including Nile tilapia, African catfish, and grey mullet at Qalubiya Governorate, beside detection of *Ps. Aeruginosa* antimicrobial sensitivity and virulence factors.

2. MATERIAL AND METHODS

2.1. Samples collection:

A total of 145 clinically diseased alive fish samples (55 Nile tilapia; 65 African catfish and 25 Grey mullet) were collected in separate sterile bags from various markets at Qalubiya Governorate, Egypt, during the period from September 2021 to May 2022, and were transported in

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icebox with minimum delay to the laboratory for isolation and phenotypic characterization of *Ps. aeruginosa.*
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2.2. Isolation and identification of Pseudomonas aeruginosa:

Small pieces of fish musculature were collected in a completely sterilized condition, inoculated in a Tryptone Soya Broth (TSB) and incubated aerobically at 25ºC for 24 hours. Then a loopful from incubated TSB was streaked onto nutrient agar, and aerobically incubated at 25°C for 24 hours. Suspected growths were picked up and purified by further subculturing on nutrient agar. The purified colonies were streaked on MacConkey agar, *Pseudomonas* agar and *Pseudomonas* Cetrimide agar, and kept for 24-48 hours at 25°C. Suspected colony was collected and kept in semisolid nutrient slope for advanced morphological and biochemical identification, represented by Triple Sugar Iron (TSI), Lysine Iron (LI), simmon citrate, urease activity, indol reaction and Vogues Proskauer (media were obtained from OXOID), according to Markey *et al*. (2013); Austin and Austin (2016).

2.3. In-vitro anti-bacterial sensitivity test for Ps. aeruginosa isolates:

Each isolated *Ps. aeruginosa* strain was tested against twelve commercial anti-bacterial discs (OXOID) following CLSI (2018). Amoxicillin (AMX), ampicillin (AM), co-trimoxazole (COT), norfloxacin (NOR), cefotaxime (CTX), ciprofloxacin (CIP), oxacillin (OX), doxycycline (DO), streptomycin(S), gentamicin (GEN), Penicillin-G (P)and tetracycline (TE) were used in the performed test.

2.4. Genotypic detection of virulence genes in Ps. aeruginosa:

Four specific primers (Metabion, Germany; table 1) were used to detect four virulence genes; exotoxin A gene (*tox*A), outer membrane lipoprotein L (*opr*L), exotoxin S gene (*exo*S) and exopolysaccharide synthesis locus gene (*psl*A). It was applied on six random isolated *Ps. aeruginosa* (two isolates from each fish sample).

Following QIA amp® DNA Mini Kit instructions (Qiagen, Germany, GmbH; Catalogue no. 51304), Emerald Amp GT PCR master-mix (Takara, Japan) with Code No. RR310A and 1.5% agarose gel electrophoreses (Sambrook *et al.*, 1989). PCR cycling conditions were followed as mentioned in table (1).

Table 1 Primers sequences, target genes, amplicons sizes and PCR cycling conditions.

Target gene		Primer sequence (5'-3')	Amplified segment (bp.)	Primary denaturation	Amplification (35 cycles)			Final extension	References
					Secondary denaturation	Annealing	Extension		
toxA	F	GACAACGCCCTCAGCATCACCAGC	396bp.	94°C/5 min.	94°C/30 sec.	55°C/ 40 sec.	72°C/ 45 sec.	72°C/ 10 min.	Matar *et al*. (2002)
	R	CGCTGGCCCATTCGCTCCAGCGCT							
oprL	F	ATG GAA ATG CTG AAA TTC GGC	504bp.			55°C 40 sec.	72°C/ 45 sec.	72°C/ 10 min.	Xu *et al*. (2004)
	R	CTT CTT CAG CTC GAC GCG ACG							
exoS	F	GCGAGGTCAGCAGAGTATCG	118bp.			55°C 30 sec.	72°C/ 30sec.	72°C/ 7 min.	Winstanley *et al*. (2005)
	R	TTCGGCGTCACTGTGGATGC							
pslA	F	TCCCTACCTCAGCAGCAAGC	656bp.			60°C 40 sec.	72°C/ 45 sec.	72°C/ 10 min.	Ghadaksaz*et al*. (2015)
	R	TGTTGTAGCCGTAGCGTTTCTG							

3. RESULTS

3.1. Clinical and postmortem examination:

The clinical examination of studied fishes showed congestion over the fish body, fins, and its bases some had eroded fins, loss of fin membrane and sometimes loss of fin rays with grey patches at tip of them. Others showed eye cloudiness, detachment of scales and skin ulceration and abdominal distention. Internally most of these fishes showed ascites; pale enlarged liver in some fishes and congested with grey patches in other ones; congested kidneys, spleen intestine that sometimes filled with yellow mucous like materials in some fishes.

3.2. Prevalence of Ps. aeruginosa isolated from examined fishes:

The prevalence of Ps. aeruginosa among the examined freshwater fishes was presented in Table (2). A total of 43Ps. aeruginosa isolates (29.7%) were identified from145 examined diseased fish samples where the prevalence rate among Nile tilapia, African catfish, and grey mullet was 25.4%, 29.2%, and 40.0% respectively.

Table 2 Prevalence of Ps. aeruginosa in the examined fishes.

Fish Samples	Number of samples	Negative samples		Positive samples	
		No.	%	No.	%
Nile tilapia (*O. niloticus*)	55	41	74.6	14	25.4
African catfish (*C. gariepinus*)	65	46	70.8	19	29.2
Mullet (*M. cephalus*)	25	15	60.0	10	40.0
Total	145	102	70.3	43	29.7

Percentage in relation to total number of each sample in each row.

3.3. Biochemical identification of isolated Pseudomonas species:

All recovered isolates grow well and showed greenish-blue, large, flat, spreading, and irregular colonies with a characteristic fruity odor on nutrient agar; Large, non-lactose fermenter (pale colonies) on MacConkey agar, bluish green colonies on Pseudomonas agar and Small, smooth with blue – green pigmented colonies on Pseudomonas

Cetrimide agar. Microscopically, they were medium-sized Gram-negative, non-capsulated, non-sporulated, straight, or slightly curved rods. All isolates showed motility on semisolid agar. So, all isolates were suspected as Ps. aeruginosa**.**

The results of biochemical identification showed that, all 43 isolates had characteristic biochemical reaction of Ps. aeruginosa, where they were positive for oxidase, catalase, citrate utilization urease, lysine decarboxylase tests without H_2S production, fermented glucose, and mannitol but they were negative for sucrose and lactose fermentation, indole, Voges-Proskauer and methyl red tests.

3.4. The antibacterial sensitivity tests for Ps. aeruginosa isolates:

The obtained findings of in-vitro sensitivity tests on the 43 isolated Ps. aeruginosa strains (Table, 3) cleared that, 97.7% of the examined isolates were resistant to ampicillin and penicillin-G; 95.3% for oxacillin; 83.7% for amoxicillin; 81.4% for cefotaxime, and 55.8% for tetracycline. Meanwhile, they showed intermediate resistance to Co-Trimoxazole (65.1%) and streptomycin (53.5%). No isolates showed complete susceptibility to any of tested drugs. The highest sensitivity was to gentamicin (79.1%) followed by norfloxacin (76.7%), ciprofloxacin (69.8%) and doxycycline (58.1%).

Table 3 In-Vitro anti-bacterial susceptibility of the isolated Pseudomonas aeruginosa strains.

Antimicrobial agents		Disk concentrations	Sensitive		Intermediate		Resistant		AA
			No.	%	No.	%	No.	%	
Amoxicillin	AMX/25	25µg	2	4.7	5	11.6	36	83.7	R
Ampicillin	AM10	10µg	0	0.0	1	2.3	42	97.7	R
Cefotaxime	CTX/30	30µg	2	4.7	6	13.9	35	81.4	R
Ciprofloxacin	CIP/5	5 µg	30	69.8	10	23.2	3	7.0	S
Co- Trimoxazole	COT/25	(1.25/23.75) µg	8	18.6	28	65.1	7	16.3	IS
Doxycycline	DO/30	30 µg	25	58.1	11	25.6	7	16.3	S
Gentamicin	GEN/10	10 µg	34	79.1	5	11.6	4	9.3	S
Norfloxacin	NOR/10	10 µg	33	76.7	8	18.6	2	4.7	S
Oxacillin	OX1	1µg	0	0.0	2	4.7	41	95.3	R
Penicillin-G	P10	10 u	0	0.0	1	2.3	42	97.7	R
Streptomycin	S/10	10 µg	4	9.3	23	53.5	16	37.2	IS
Tetracycline	TE/30	30 µg	5	11.6	14	32.6	24	55.8	R

No.: Number of isolates AA: Antibiogram activity%: Percentage in relation to total number of isolates n=43

3.5. PCR detection of some virulence genes in Ps. Aeruginosa isolates:

PCR detection of toxA, oprL, exoS and pslA virulence genes in six *Ps. Aeruginosa* isolates showed that toxA; oprL and pslA virulence gene were detected in three out of them isolates (two from *C. gariepinus* and one from *O. niloticus* fish) giving products of 396 bp, 504 bp, and 656 bp, respectively but not detected in *M. cephalus* fish (Figs. 1-a and 1-b and Fig.2-b). while, exoS gene was detected in five isolates (two from *M. cephalus* and *C. gariepinus* and one from *O. niloticus* fish samples)with product of 118 bp (Fig. 2- a).

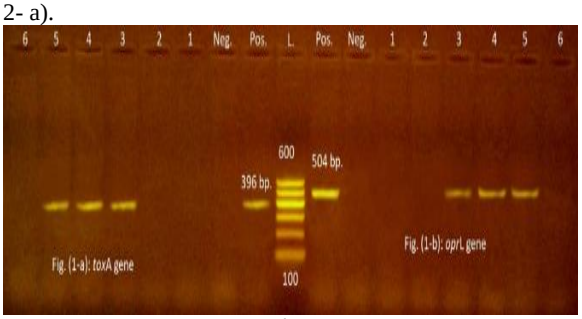

Figure (1-a) Exotoxin A (*tox*A) gene of isolated *Ps. aeruginosa* among examined freshwater fishes. Lane L: 100-600 bp. DNA Ladder, Neg.: Negative control. (*S. aureus* ATCC25923), Pos.: Positive control (*Ps. aeruginosa* form Ahri. at 396 bp.), Lane 1-2: Negative *Ps. aeruginosa* at 396 bp. (*M. cephalus*), Lane 3- 5: Positive *Ps. aeruginosa* at 396 bp. (3 and 4 *C. gariepinus* and 5 *O. niloticus*), Lane 6: Negative *Ps. aeruginosa* at 396 bp. (*O.niloticus*).

Fig. (1-b): Outer membrane lipoprotein L (*opr*L) gene of isolated *Ps. aeruginosa* among examined freshwater fishes. Lane L: 100-600 bp. DNA, Neg.: Negative control. (*S. aureus* ATCC25923), Pos.: Positive control (*Ps. aeruginosa* form Ahri. at 504 bp.), Lane 1-2: Negative *Ps. aeruginosa* at 504 bp. (*M. cephalus*), Lane 3- 5: Positive *Ps. aeruginosa* at 504 bp. (3 and 4 *C. gariepinus* and 5 *O. niloticus*), Lane 6: Negative *Ps. aeruginosa* at 504 bp. (*O. niloticus*)

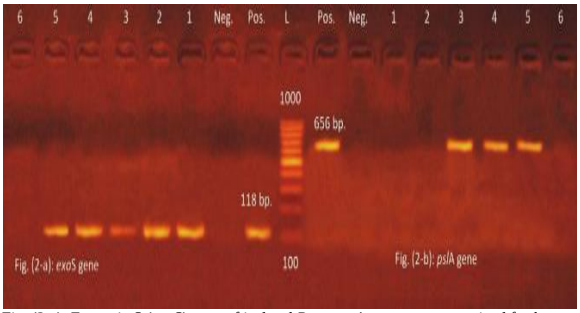

Fig. (2-a): Exotoxin S (*exo*S) gene of isolated *Ps. aeruginosa* among examined freshwater fishes. Lane L: 100-1000 bp. DNA, Neg.: Negative control. (*S. aureus* ATCC25923), Pos.: Positive control (*Ps. aeruginosa* form Ahri. at 118 bp.), Lane 1-5: Positive *Ps. aeruginosa* at 118 bp. (1&2 *M. cephalus;* 3 and 4*C. gariepinus* and 5 *O.niloticus*), Lane 6: Negative *Ps. aeruginosa* at 118 bp. (from *O.niloticus*)

Fig. (2-b): Exopolysaccharide synthesis locus gene (*psl*A) gene of isolated *Ps. aeruginosa* among examined freshwater fishes. Lane L: 100-1000 bp. DNA, Neg.: Negative control. (*S. aureus* ATCC25923), Pos.: Positive control (*Ps. aeruginosa* form Ahri. at 656 bp.), Lane 3-5: Positive *Ps. aeruginosa* at 656 bp. (3 and 4*C. gariepinus* and 5 *O.niloticus*), Lane 1, 2 and 6: Negative *Ps. aeruginosa* at 656 bp. (1 and 2 *M. cephalus* and 6 *O.niloticus*).

4. DISCUSSION

Pseudomonas spp., especially *Ps. aeruginosa*, have been recorded as one of the most vigorous causes of ulcerative infections among fish populations leaving high mortalities, high economic losses and decreases fish farm efficiencies (Shahrokhi *et al*., 2022), beside their zoonotic impact on gastrointestinal and extra intestinal infections (Benie *et al*., 2017andAbdulhaq *et al*., 2020).

The bacteriological examination in the present study detected 43 *Ps. aeruginosa* isolates (13.8%) from 145 diseased fish samples. The pathogen was recorded among

examined fishes as 14/55 (25.4%) from *O. niloticus*;19/65 (29.2%) from *C. gariepinus* and from 10/25 (40.0%) *M. cephalus*. These records came in harmony with those obtained by Hanna *et al*. (2014); Ibrahim *et al*. (2016); Salem *et al*. (2018) and Abd El Tawab *et al*. (2016 and 2019). Meanwhile, they disagree with those of Abd El-Maogoud *et al*. (2021) and Mamdouh *et al*. (2022) who recorded higher incidences 47.3% and 65.0%, respectively and with Shahrokhi *et al*. (2022) who recorded lower incidence 5.0%.

The macroscopic characteristics of the colony culture, Gram reaction and the biochemical profile of *Pseudomonas* species isolated such as the fermentation of certain sugars or enzymatic reaction as protease; lipase; extracellular pigmentation production and was like those previously reported by Markey *et al*. (2013); Hanna *et al*. (2014); Abd El Tawab *et al*. (2016); Austin and Austin (2016) and Shahrokhi *et al*. (2022).

The obtained findings of in-vitro sensitivity tests on the 43 isolated *Ps. aeruginosa* strains (Table 3) cleared that the examined isolates were highly resistant to ampicillin and penicillin-G, oxacillin, amoxicillin, cefotaxime, and tetracycline. Meanwhile, they showed intermediate resistant to Co-Trimoxazole and streptomycin.

No isolates showed complete susceptibility to any of tested drugs. The highest sensitivity was to gentamicin (79.1%) followed by norfloxacin (76.7%), ciprofloxacin (69.8%) and doxycycline (58.1%). Nearly compatible results were previously reported by Hanna *et al*. (2014); Roy *et al*. (2014); Abd El Tawab *et al*. (2016) and Shahrokhi *et al*. (2022).

The pathogenicity of *Ps. aeruginosa* is mostly due to its ability to produce large number of virulence genes. These genes participate in specific ways in the infection process, and each gene may affect host cell signal transduction in ways which enhance the spread of infection (Nafee, 2012; Nowroozi *et al*., 2012 and Markey *et al*., 2013).

Exotoxin A gene (*tox*A) is the most extracellular toxic protein of the pathogenic *Ps. aeruginosa* for animals, fish, and human. It prevents protein-synthesis in the cell (Michalska and Wolf, 2015; Aljebory et *al*., 2018). The PCR amplification findings of *tox*A gene cleared that, it was amplified in three out of six studied *Ps. aeruginosa* isolates giving products of 396 bp. These results are in constancy with those obtained by Matar *et al*., (2002); Nafee (2012); Khattab *et al*., (2015); Abd El Tawab *et al*., (2016 and 2019); Abd El-Maogoud *et al*., (2021) and Shahrokhi *et al*., (2022).

The *opr*L is a vigorous necrotic factor for cells that has been reported to have a significant role in the bacterium interaction with the surrounding conditions, as well as the inherent anti-bacterial resistance of *Ps. aeruginosa* (Markey *et al*., 2013). The obtained PCR result for amplification of *opr*L gene in *Ps. aeruginosa* strains revealed that, it was amplified in three out of six studied *Ps. aeruginosa* isolates giving products of 504 bp. Nearby findings were previously reported by Xu *et al*. (2004); Abdullahi *et al*. (2013); Khattab *et al*. (2015); Abd El Tawab *et al*. (2016 and 2019); Abdulhaq *et al*. (2020) and Abd El-Maogoud *et al*. (2021).

In addition, the *exo*S gene is a lethal factor causing cellular deaths as it decreases DNA synthesis and viability of the infected cells (Fadhil *et al*., 2016).The molecular findings for amplification of *exo*S gene in *Ps. Aeruginosa* isolates showed amplification of *exo*S gene in five out of six studied isolates; giving product of 118 bp. Similar findings were previously recorded by Khattab *et al*. (2015); Abd El Tawab *et al*. (2016 and 2019); Benie *et al*. (2017) and Shahrokhi *et al*. (2022).

The attachment and production of extracellular polysaccharides are essentials to start biofilm genesis in the bacteria. The *psl*A gene is key part of the polysaccharide synthesis locus (*psl*) accountable for the secretion of extracellular polysaccharide in *Ps. aeruginosa*. The PCR amplification findings of *psl*A gene of *Ps. Aeruginosa* showed that the *psl*A gene was amplified in three out of six studied *Ps. aeruginosa* isolates giving products of 504 bp. Similar detection of *psl*A gene in antibiotic resistant with biofilm formation Ps. *aeruginosa* strains isolated from human, animals and environment were recorded by Ghadaksaz *et al*. (2015); Nader *et al*. (2017); Abdulhaq *et al*. (2020); Madaha *et al*. (2020); Ugwuanyi *et al*. (2021) and Schimmunech *et al*. (2022).

5. CONCLUSION

The obtained results concluded that *ps. aeruginosa* is important pathogen causes Pseudomonas septicemia in fish. Multiple antibiotic resistances are widely spread among 43 *Ps. aeruginosa* isolates. The detection of virulence genes in them indicates their pathogenicity for fish, which represent a potential risk for fish - originated food poisoning and increasing prevalence of community acquired infection. However, gentamicin; norfloxacin; ciprofloxacin and doxycycline could be used for controlling Pseudomonas septicemia in fish.

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