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The Transmission of *Pseudomonas putida* in Some *Tilapia* species in Egypt: The Potential Role of *Clinostomum* Infestation

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ABSTRACT

Clinostomum (a digenetic trematode) infests tilapia and causes several disease problems. Moreover, *Clinostomum* has zoonotic importance. *Pseudomonas putida* is regarded as one of the significant pathogens that have threatened freshwater fishes resulting in high mortality and severe economic loss. This study investigates the phenotypic and genotypic characterization of *P. putida* recovered from both fish and their *Clinostomum* that may bond the *Clinostomum* infestation to the transmission of *P. putida* in *Tilapia* fishes. Fifty *P. putida* isolates from freshly dead moribund fishes and their *Clinostomum*, from the Nile River at Al Bahr Al Aazam, Giza, were studied. Also, 5 isolates were retrieved from the water. Biochemical, antibiogram, species-specific PCR and phylogenetic analysis of 16S rRNA gene were performed. Also, Water physicochemical analysis was done. The analysis of water showed bad water quality in summer. *P. putida* isolates from fish, *Clinostomum* and water showed the exact antimicrobial susceptibility profile. The phylogenetic analysis of partial *16S rRNA* gene sequence revealed that *P. putida* isolates from fish, *Clinostomum* and water showed 100% similarity with each other. This result, combined with our isolation of viable *P. putida* from *Clinostomum*, proposes the transmission of this serious pathogen through the parasite. In conclusion**,** the high *Clinostomum* infestation could play a significant role in the transmission and high prevalence of *P. putida* in *Tilapia* fishes in the Nile River at Al-Bahr Al-Azam, Giza.

INTRODUCTION

The Nile River is the major freshwater resource of many aquatic food organisms in Egypt **(Mahmoud *et al*., 2014)**. The two main challenges that limit aquaculture development are fish diseases and water pollution **(Austin and Austin, 2012).** In Egypt, *streptococcosis*, *pseudomonas* and *aeromonas* septicemia have the highest risk impact on the Nile tilapia *(Oreochromis niloticus)* **(Eissa, 2016)**. The frequency and severity of diseases are directly connected to the hygiene of the water **(Hossain *et al*., 2007).** Poor water quality (lower dissolved oxygen, inadequate temperature and pH values, and high levels of organic matter in the water) creates a favorable environment for the

development of pathogenic agents, primarily fish ecto-parasites **(Paulista *et al.*, 2009).** Parasites have been identified as an important obstacle in aquaculture since they induce significant mortalities and slow growth rates **(Iwanowicz, 2011).** In Egypt, parasitic diseases account for nearly 80% of fish diseases**.** This might be a result of prolonged warm weather and the abundance of natural food, as well as the presence of intermediate hosts like mollusks and water insects **(Eissa, 2002)**. At Maryotia stream in Egypt, which is susceptible to numerous sources of pollution through the disposal of sewage materials as well as improperly handled inorganic and organic chemical wastes, (**Mahmoud *et al.*, 2014)** observed mass mortalities among *O. niloticus* that have a 100% parasitic infestation rate of Copepods, monogenea, protozoa, trematode larvae, and *Acanthocephala*. Stress from parasitism reduced fish resistance to bacterial co-infections and opened a door for secondary invaders (**Bowers *et al*., 2000**).

Digenetic trematode infections are the most prevalent in freshwater fish with a multi-stage life cycle in various hosts **(Aghlmandi *et al.*, 2018; Faruk, 2018; Calhoun *et al.*, 2019).** *Clinostomum* metacercariae infect tilapia and cause several disease problems. These digenetic metacercariae have been encysted in the submucosa of the buccal cavity, the gill chamber, the operculum, the muscles, abdominal cavity, mesentery and viscera **(Chung *et al.*, 1995),** causing low weight and growth, unusual host behaviors, low marketability and death especially in case of heavy infestations **(Wang *et al.*, 2017)**. Moreover, *Clinostomum* have zoonotic importance in the transmission of yellow grub disease to humans via ingesting raw or improperly cooked fish resulting in a clinical syndrome called halzoun **(Shamsan and Al-jobory, 2018).**

P. putida is regarded one of the significant bacterial pathogens that have threatened fresh and marine fishes resulting in high mortality and severe economic loss **(El-Barbary and Hal, 2017)**. *P. putida* persist harsh environment by utilizing nutrients from dead cells and its ability to form biofilms that act as a useful place for it **(Lynch, 1990; Yadav *et al.*, 2019)**. *P. putida* was isolated from *O. niloticus* in Egypt, and caused exophthalmia, ascites, and ulceration on the fish body **(Atwa, 2007; Salama and Gharib, 2009; Enany *et al*., 2019).**

Laboratory parasitism of *Tilapia* with *Gyrodactylus niloticus* (a monogenetic trematode) was found to harbor live *Streptococcus iniae* after the experimental infection, indicating that *G. niloticus* may transmit *S. iniae* from fish to fish **(Shoemaker *et al.*, 2008).** In our previous study **(El kabany *et al.*, 2023a),** we have recorded natural concurrent *Pseudomonas* infection of 56.41% with *Clinostomum* infestation. Also, mixed infection of cultured *O. niloticus* with *P. putida* and external parasitic protozoa was previously reported **(Salama and Gharib, 2009)**. Therefore, the present study was designed to investigate the phenotypic and genotypic characterization of *P. putida* recovered from both fish and their *Clinostomum* that may bond the *Clinostomum* infestation to the transmission of *P. putida* in *O. niloticus* and *Tilapia zilli* fish in freshwater environment.

MATERIALS AND METHODS

1. **Fish sampling**

A total of 93 (45 in spring and 48 in summer) freshly dead infested *O. niloticus* and *T. zilli* fishes were collected from the freshwater environment of the Nile River at Al Bahr Al Aazam, Giza in the spring and summer of 2019. Samples were properly transferred to the laboratory, after identification, for parasitic and bacterial isolation.

2. **water analysis**

Physicochemical parameters of water samples were analyzed following **(El kabany *et al.*, 2023b)**

3. **Bacterial isolation**

3.1. Fish

Fifty *pseudomonas spp* isolates (20 in spring and 30 in summer) from *O.niloticus* and *T.zilli* fish concurrently infested with *Clinostomum spp* from the Nile river at Bahr Azam, Giza were used **(El kabany *et al.*, 2023a)**. Pure colonies were streaked on the selective media *Pseudomonas* Agar Base (HIMEDIA, India) supplemented with cetrimide and nalidixic acid (HIMEDIA, India) and incubated at 28 °C for 24 hrs.

3.2. Clinostomum metacercaria

Metacercariae were extracted with fine-tipped sterile forceps then washed three times using phosphate buffer saline and surface-sterilized with 70% Ethanol (Fig.1). Each five metacercariae were pooled and homogenized and serially diluted in a sterile physiological solution **(Delhoumi *et al*., 2020).**

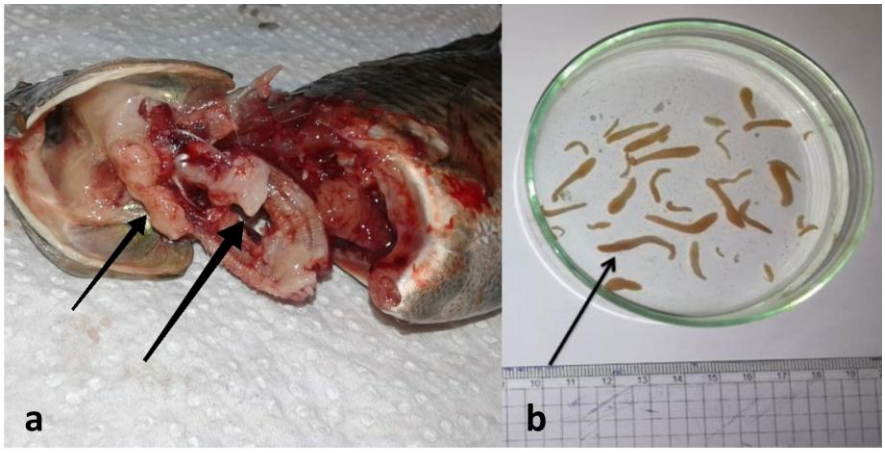

Fig. 1. a) Infested *T. zilli* with *Clinostomum* metacercariae in the buccal cavity tissues (black arrows), **b)** Excysted *Clinostomum* metacercariae

All samples from *clinostomum* pools were then inoculated into brain heart infusion broth (BHIB; HIMEDIA, India) and incubated at 28°C for 24 h**.** A loopful of the obtained

broth culture was streaked on the *Pseudomonas* Agar Base selective media and incubated 28 °C for 24 h.

3.3. Water

Three water samples were taken both in spring and summer. Samples were aseptically ten-fold serially diluted **(APHA, 2005)**, then streaked on the supplemented *Pseudomonas* Agar Base media and incubated 28 °C for 24 h. Pure colonies were stored in BHIB + 15% (vol/ vol) glycerol at -20 °C.

4. Bacterial Identification

4.1. Morphological and biochemical examination

Pure cultures of isolated bacteria were identified biochemically according to **(Eissa *et al.*, 2010)**

4.2. Antimicrobial susceptibility testing

antimicrobial susceptibility testing of *Ps. putida* isolates was operated using Disk diffusion method on Mueller–Hinton agar (Oxoid, UK) with 2% (w/v) NaCl (**CLSI**, **2020**). Results were classified as susceptible (S), resistant (R), or intermediately resistant (I), according to (**CLSI**, **2020**). The isolates were tested against 12 antimicrobials: Ceftriaxone (CTR; 30µg), Ciprofloxacin (CIP; 5µg), Tobramycin (TOB; 10µg), Amikacin (AK; 30µg), Ampicillin (AMP; 10µg), Imipenem (IPM; 10 µg), Ceftazidime (CAZ; 30µg), Cefoxitin (CX; 30µg), Gentamicin (GEN; 10 µg), Cefazolin (CZ; 30µg), Levofloxacin (LE; 5µg), and Norfloxacin (NX;10 µg) (HIMEDIA, India).

4.3. Genotypic identification

DNA was extracted according to **(Devi *et al.*, 2009)**. *Ps. putida* was identified by species-specific PCR using the 16S rRNA gene according to **(Altinok, 2011)**. The PCR products of 3 isolates from fish and its *clinostomum*, and from water were sequenced according to **(El kabany *et al.*, 2023b)**

RESULTS

1. Water analysis

The physiochemical analysis of water showed worse water quality in summer than spring for temperature, dissolved oxygen, pH, ammonia, nitrite, and nitrate (Table 1).

Table 1. Mean of physicochemical analysis of water samples collected from Bahr Aazam, Giza

Water quality parameter	**Season spring**	**Season summer**	**Permissible limits***
DO (mg/L)	6.2	4.7	> 5 mg/L
Temp.(°C)	24.7	30.7	
pH	8.7	9.1	6.5–9
Ammonia (mg/L)	0.42	0.56	0.187-0.473
Nitrite (mg/L)	0.07	0.09	0.06 mg/L
Nitrate (mg/L)	5.29	6.69	2.9 mg/L

* Recommended limits as reported by (**CCME**, **2011**)

2. Bacterial isolation

In spring, 20 isolates were retrieved from both fish and their *clinostomum* pool (20/45, 44%), while in summer, 30 isolates were purified from both fish and their *clinostomum* pool (30/48, 63%). From water samples, 5 isolates were isolated (2 from spring samples (67%), and 3 from summer samples (100%).

3. Bacterial Identification

3.1. Morphological and biochemical examination

On supplemented *pseudomonas* base agar plates, small flat straw colonies of *P. putia* were observed (Fig. 2). *P. putida* isolates from fish, *Clinostomum* and water showed the same biochemical characteristics; motile, Gram-negative short rods, oxidase positive, catalase positive, indole negative, methyl red negative, positive glucose fermentation, and negative gelatin liquefaction.

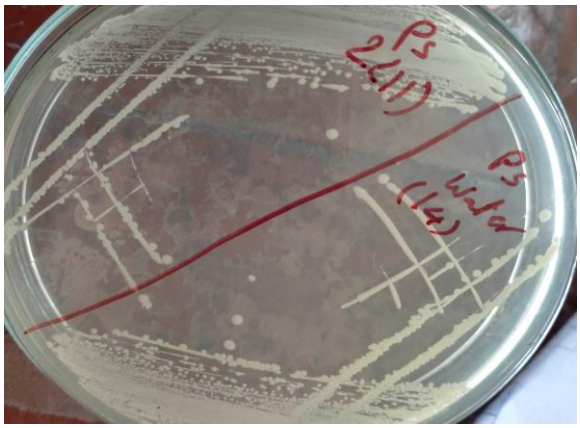

Fig. 2. Small flat straw *P. putida* colonies on *Pseudomonas* Agar Base media

3.2. Antimicrobial susceptibility testing

Table (2) shows the antimicrobial susceptibility of *P. Putida* isolates isolated from fish and their *clinostomum* (n = 50), and from water (n = 5). Isolates showed 100% susceptibility profile agreement for all tested antibiotics. All isolates were sensitive to Tobramycin, Amikacin, Imipenem, Gentamicin, Levofloxacin and Norfloxacin. On the other hand, they were all resistant to Ceftriaxone, Ampicillin, Ceftazidime, Cefoxitin, Ciprofloxacin and Cefazolin.

Table 2. Antimicrobial resistance of *P. putida* isolates from fish (*n* = 50), *Clinostomum spp* (*n* = 50) and water (*n* = 5) at Al-Bahr Al-Azam, Giza

Antibiotic	**Fish isolates**		***Clinostomum* isolates**		**Water isolates**	
Profile	**S**	**R**	**S**	**R**	**S**	**R**
CTR 30		50 (100%)		50 (100%)		5 (100%)
CIP 5		50 (100%)		50 (100%)		5 (100%)
AMP 10		50 (100%)		50 (100%)		5 (100%)
CAZ 30		50 (100%)		50 (100%)		5 (100%)
CX 30		50 (100%)		50 (100%)		5 (100%)
CZ 30		50 (100%)		50 (100%)		5 (100%)
TOB 10	50 (100%)		50 (100%)		5 (100%)	
AK 30	50 (100%)		50 (100%)		5 (100%)	
IPM 10	50 (100%)		50 (100%)		5 (100%)	
GEN 10	50 (100%)		50 (100%)		5 (100%)	
LE 5	50 (100%)		50 (100%)		5 (100%)	
NX 10	50 (100%)		50 (100%)		5 (100%)	

3.3. Genotypic characterization

P. putida was confirmed by species-specific PCR targeting the 16s rRNA gene. All tested isolates showed the characteristic amplicon size of 380 bp (Fig. 3).

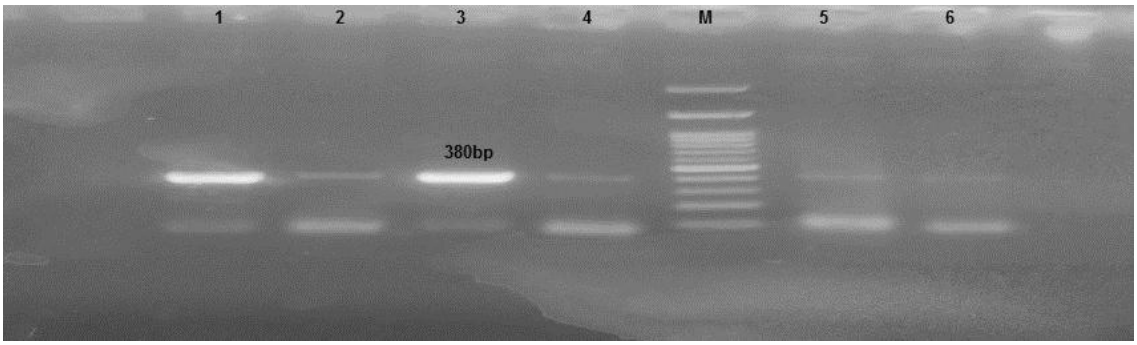

Fig. 3. PCR of the partial 16S rRNA gene sequence at 380 bp of *P. putida*. (M) 100 bp ladder

After sequencing, the obtained sequences were submitted in the Gene Bank https://blast.ncbi.nlm.nih.gov/Blast with accession numbers (MZ452332), (MZ452334), (OK235621) for fish, *Clinostomum* and water isolates, respectively. The derived sequences were clustered with their relevant sequences using neighbor-joining phylogenetic tree. The phylogenetic analysis showed our *P. putida* isolates were embedded among other *P. putida* isolates with >99% strong bootstrap value (Fig. 4). *P. putida* isolates from fish, *Clinostomum* and water showed 100% similarity with each other. Our isolates showed identity of 98.84% - 95.84% with *P. putida* isolated from occupied Palestine and India.

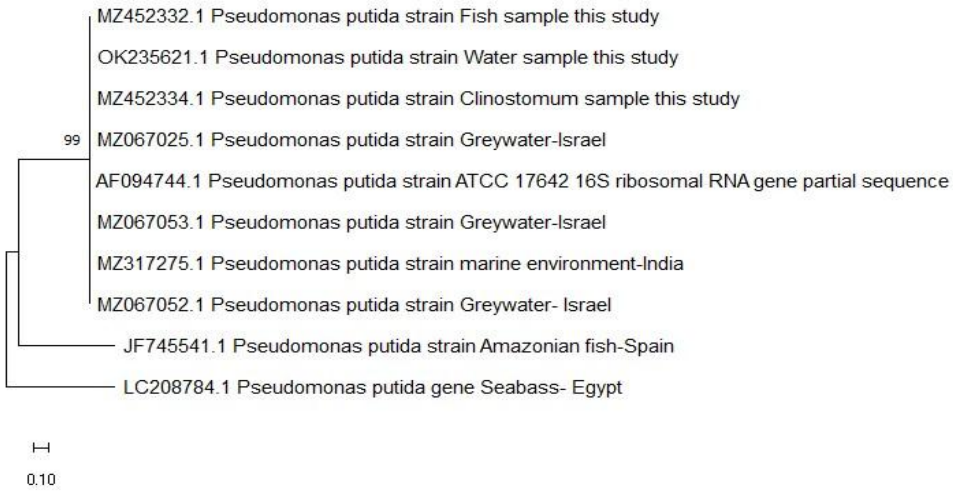

Fig. 4. Neighbor-joining phylogenetic tree showing the relation between fish, *Clinostomum* and water isolates' sequences among other bacteria

DISCUSSION

Multiple disease agents, such as parasites, bacteria, or a combination of them, truly exist in intensive fish farming resulting in high disease losses **(Shoemaker *et al.*, 2008)**. The current study investigated the probable correlation between *clinostomum* invasions and *P. putida* infection transmission in *O. niloticus* and *T. zilli* in their natural environment.

Water quality deterioration creates favorable conditions for parasitic proliferation and pathogenic infections that impair fish production **(Paulista *et al.*, 2009; Ojwala *et al.*, 2018**). In our study, low DO levels (4.7 mg/L), high temperature (30.7°C), high levels of ammonia (0.56 mg/L), nitrite (0.09 mg/L) and nitrate (6.69 mg/L) were recorded in summer. Water temperature not only affects parasites directly, but also indirectly by favoring presence of intermediate hosts (**Khan**, **2012**; **Lõhmus and Björklund**, **2015**). The increase in Ammonia may be pointed to the domestic, industrial effluents and agricultural drainage water (**Elghobashy *et al.*, 2001**) while the appearance of NO3 in concentrations > 5 ppm is reflecting unsanitary conditions (**Uqab *et al.* 2017**)in the Nile water. The recorded physico-chemical levels of water, during summer in our study, represent the ultimate stress conditions that resulted in high concurrent prevalence of 63% in summer compared to 44% in spring. Our results agree with (**Lagrue *et al.*, 2011; Ojwala *et al.*, 2018**) who reported that low water quality has a positive influence on the occurrence of parasitic populations and pathogenic communities. Also, we partially agree with (**Waruiru *et al.*, 2020)** who stated that the parasitic prevalence was positively correlated with ammonia free nitrogen, and nitrates, and that the level of dissolved oxygen demonstrated a significantly positive correlation to the occurrence and intensity of *Clinostomum* The occurrence of poor water quality parameters, particularly during the summer, may be caused by climatic changes combined with high levels of pollution at Giza as a result of dense populations, traffic, industries, and various anthropogenic activities that degrades water quality. Therefore, we disagree with (**Shafi *et al.*, 2015**) who considered that the changes in the water quality due to anthropogenic pollution have not led to an increase in the parasitic load.

Concerning the phenotypic and biochemical characters of *P. putida*, all isolates from fish, *Cinostomum*, and water exhibited the same characteristics. The morphological and biochemical characteristics of our isolates were very similar to those of *P. putida*, as described by **(Buller, 2004; Austin and Austin, 2012)**.

In the present study, all *P. putida* isolates were sensitive to Tobramycin, Amikacin, Imipenem, Gentamicin, Levofloxacin and Norfloxacin. On the other hand, they were all resistant to Ceftriaxone, Ampicillin, Ceftazidime, Cefoxitin, Ciprofloxacin and Cefazolin. These results were in complete accordance with **(El-Barbary and Hal, 2016; Ginovyan *et al.*, 2017; Enany *et al.*, 2019)** who observed that *P. putida* isolates were sensitive to Norfloxacin and Tobramycin, while resistant to cefazolin. Our results

were slightly differed from **(Urku, 2021; Alzahrani *et al.*, 2022)** who found that isolates of *P. putida* were sensitive to Ciprofloxacin and resistant to Ampicillin. At the present, rivers are highly contaminated with antibiotic-resistant bacteria, transferred from sewage drainage, especially from hospitals, agricultural drainage, slaughterhouses, clinics, and animal husbandries (**Djenadi**, **2017**). Recently, (**Zaki *et al.*, 2023**) found antibiotic-resistant bacteria in the Nile River's raw and tap water in the Dakahlia Region of Egypt due to urbanization, accumulation of microbial contamination during different water transfers from source to homes, misuse and increased exposure to antibiotics in human treatment, in poultry, and livestock farms and agriculture, which may pose a serious ecological risk to the waters and public health. Therefore, careful use of antibiotics should be applied to counteract the rising problem of multidrug resistant *P. putida* that is both survive tough environments and zoonotic.

PCR identification of *P. Putida* was confirmed by partial sequencing of the -16S rRNA gene. The phylogenetic analysis revealed 100% similarity of *P. putida* isolates from fish, *clinostomum* and water. This result, combined with our isolation of viable *P. putida* from *Clinostomum*, proposes the transmission of this serious pathogen through the parasitic and improves the default theory of the parasite being only the stress factor that opens the skin and lowers the immunity of the fish for the secondary bacterial infections.

In addition to the deterioration of water quality, the high *Clinostomum* infestation could play a significant role in the transmission and high prevalence of *P. putida* in *Tilapia* fishes in the Nile River at Al-Bahr Al-Azam, Giza.

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