Alfarama Journal of Basic & Applied Sciences

Faculty of Science Port Said University

https://ajbas.journals.ekb.eg

ajbas@sci.psu.edu.eg

http://sci.psu.edu.eg/en/

ISSN 2682-275X

July 2020, Volume 1, Issue 2

DOI:10.21608/AJBAS.2020.23319.1009

Submitted: 17-02-2020

Accepted: 07-06-2020

Pages: 60-77

Evaluation of bacterial quality of *Oreochromis niloticus* samples of Lake Manzala, Egypt.

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ABSTRACT

The present study aimed to investigate bacterial quality of tilapia fish (*Oreochromis niloticus*) and evaluate the hygienic health hazard of fish contaminated with some food borne pathogens. Bacteria were isolated from intestine, gills and flesh of *Oreochromis niloticus* collected from Lake Manzala during two seasons; winter and summer, 2017. Microbiological studies was applied for all samples as total viable bacteria, total coliform, faecal coliform, faecal streptococcus and examined for the presence of *Pseudomonas* sp. Results show that total viable bacterial count mean was highest in intestine 222 x 10^4 $CFUg^{-1}$ in summer. Total coliform count mean was highest in intestine 425 x 103 $CFUg^{-1}$ in summer. Faecal coliform count mean was highest in gills 90 x 10^2 $CFUg^{-1}$ in summer. Faecal streptococcus detected in fish organs only in summer. *P. aeruginosa* count mean was highest in intestine 185 x 10^2 $CFUg^{-1}$ in summer. Molecular identification of suspected colonies was as DNA extraction, PCR technique using ISSR protocol and sequencing, using the nucleotide sequences of the genes for 16S rRNA revealed different strains of *Pseudomonas aeruginosa*. All isolates were tested for resistance to 10 groups of antibiotics namely Tetracycline (10µg), Gentamicin (10µg), Ampicillin (10µg), Trimethoprim/Sulphamethoxazole (25µg), Vancomycin (30µg), Erythromycin (15µg), Ciprofloxacin (30µg), Chloramphenicol (30µg), Amoxicillin (10µg) and Rifampicin (5µg) using Kirby-Bauer disc diffusion method. The results showed high frequency of multi-drug resistance to many antibiotics, particularly penicillin, ampicillin and chloramphenicol. Therefore this fish species caught from the Lake Manzala may pose health hazards to human and the whole ecosystem.

Keywords

Pseudomonas aeruginosa, Oreochromis niloticus, Multidrug resistant, Lake Manzala

1. INTRODUCTION

Bacteria are imperative microorganisms in lakes and ponds, whereby, some are beneficial, others are not. Beneficial bacteria are that occur naturally in water , streams, ponds, etc. are responsible for breaking down organic waste and ammonia from fish waste, reducing nitrite and nitrate and as sources of food,

subsequently play a vital role in balancing lake ecosystems and also in fish production. Non-beneficial bacteria cause offensive smell to lakes additionally infections in fishes [1]. The reservoir of bacterial resistance genes in the environment is due to a blend of naturally occurring resistance and those present in water, fish, animal and human waste and the selective effects of pollutants, which can co-select for mobile genetic elements carrying multiple resistant genes [2].

Lake Manzala is the largest and the most productive lake of the northern Egyptian coastal lakes and the foremost productive for fisheries. As shown in figure1, it is connected to the Mediterranean Sea through the outlet of Boughaz El-Gamil. The western and southern shores of the lake have many agricultural water drains pour into the lagoon such as Bahr El-Baqar, Hadous, Ramsis, El-Serw, Gamalya, and Faraskur. Moreover, in the last six decades Lake Manzala subjected to various threats and became contaminated with heavy metals released from domestic, industrial, mining and agricultural effluents. Heavy metals are especially toxic due to their ability to bind with proteins and prevent DNA replication [3]. The lake has been gradually transformed from a largely marine or estuarine environment to a eutrophic nearly fresh water system which can have significant negative ecological, health, social and economic impacts on human [4]. These result in poor water quality and reduction in the lake productivity of fish [5].

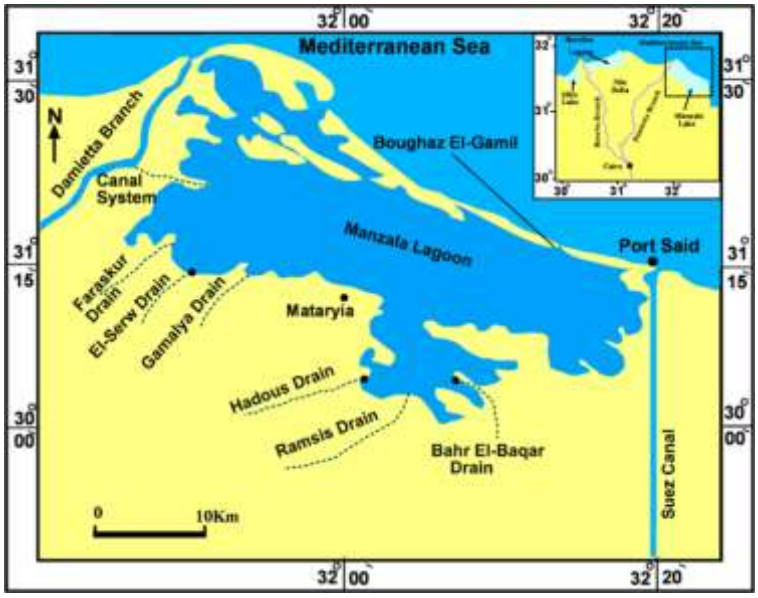

Fig. 1. Location map of Lake Manzala [6].

Lake Manzala represents 32% of the lakes fish production in Egypt and 44% of the Northern lakes [7]; while its production of tilapia constitute more than 65% of all fish production [8,9]. *Oreochromis niloticus* L. (tilapia) is the main species of freshwater fishes and one of the most popular, cheapest, and available fish for all Egyptians. It can survive in awful environmental conditions because their resistance to disease is physically powerful. The species is favored among aquaculturists due to its ability to tolerate a wide range of environmental conditions, fast growth, successful reproductive strategies, and ability to feed at different trophic levels [10, 11]. Composition of micro-floral on newly catch fish depends on the microbial content of the water in which the fish live and the food entering the digestive tract. Fish microflora includes bacteria in fish such as *Pseudomonas, Alcaligens, Vibrio, serratia* and *Micrococcus* [12].

Attention has been drawn to the possible development of antibiotic-resistant microorganisms, and research in this area has mainly focused on fish pathogens. As the fish are used for human consumption, the development of antibiotic resistance in pathogens could pose a health risk to the consumer. Resistance towards antibiotics is often associated with plasmids and transfer of R-plasmids to non-resistant strains may occur [13].

Pseudomonas sp. may become involved in the disease processes and act as secondary invaders of fish compromised by the pathogens or other factors [14]. In previous studies, it was reported that *Pseudomonas*

spp. reflected very high distribution in Lake Manzala [14-16]. In the pathogenicity test, the most pathogenic bacteria for tilapia fish was *Pseudomonas* spp., causing greater than 70%, mortality within 96 hr [15].

The genus *Pseudomonas* is a group of Gram-negative, motile rods, aerobic that are catalase and oxidase-positive [17]. The genus includes the species with function of ecological and health related importance. Some species exhibit pathogen suppressing functions [18]. Several species of these bacteria produce antibiotics which suppress phytopathogens growth [19]. Moreover, others added that surface colonization by *Pseudomonas* spp. is accompanied with induction of host plant defence [20]. *Pseudomonas* species are important spoilage organisms in many chilled food products, such as milk, chicken and meat [21]. In addition, *Pseudomonas* species are frequently associated with fish and have been isolated from skin, gills and intestine.

Pseudomonas aeruginosa is opportunistic human pathogen causing infection ranging from neonatal sepsis to burn sepsis, and lung infections [22]. *P. aeruginosa* is naturally resistant to many widely used antibiotics. Resistance in part is thought to be the result of an impermeable outer membrane and the production of extracellular polysaccharides .Individual strains may be resistant to antibiotics to which the species is generally susceptible. For this reason, antibiotic resistance patterns should not be relied on for species verification, but should be assessed on a case-by-case basis [23]. *P. aeruginosa* was previously isolated from tilapia catched from Qaroun and Wadi-El-Rayan lakes, Egypt [24]. Antibiotic resistant bacteria have increased in environment, creating a worldwide impact on both public and environmental health. It was suggested that antibiotic resistance may be related to water quality declined due to untreated sewage from rural areas, domestic wastewater, intensive agricultural and industrial activities, livestock farms, and urban runoff [25].

The importance and pathogenesis of *Pseudomonas* spp. and their widespread were the main reasons for this paper. The objective of this study is to assess the bacterial quality of tilapia fish as the most common fish in Lake Manzala and to investigate widespread of *Pseudomonas* sp in tilapia. Moreover, attempt was made to elucidate the multiple antibiotic resistance patterns in isolated *Pseudomonas aeruginosa* and evaluate the hygienic health hazard of fish contaminated with some food borne pathogens.

2. MATERIALS AND METHODS

2.1 Samples collection

Oreochromis niloticus fish samples were collected from fishermen in Lake Manzala during winter and summer seasons of 2017. Fish samples were transported immediately within sterile plastic bags in ice box to the laboratory, where samples of different organs were sorted for the bacteriological examination.

2.2.Preparing samples for bacteriological examination

The weight of the collected fish samples ranged from 53 g to 112 g, with length ranged between 14 cm and 17.5 cm. Fish skin was washed by 70% ethanol to reduce incidental organisms, then the flesh, gills and intestine were separated and taken with sterile scissors. From each fish, 1 g of each tested organ was removed and suspended in 99 ml of sterile saline (0.85% w/v NaCl) and homogenized under complete aseptic conditions. The suspension was serially diluted then 0.1 ml of the diluted solutions were spread on all media; Endo agar medium, Slanetz and Bartley medium and *Pseudomonas* agar base medium, except for nutrient agar medium plate, 1 ml of serially diluted solutions were cultured by the pour-plate method. The inoculated plates were cultured. Three replicates of each specimen were prepared.

2.3 Incubation conditions

For total aerobic bacterial counts in gills, flesh and intestine of fish samples, nutrient agar medium were used and incubated at 37^{o}C for 24 h. For total coliform, Endo agar medium were used and incubated at 35^{o}C

for 24 h. Other plates with Endo agar medium incubated at 44 ^{o}C for 24 h to detect faecal coliform. For faecal streptococci bacteria, Slanetz and Bartley medium used and incubated firstly at 35 -37 ^{o}C for 4 h then at 44^{o}C for 48 h to detect faecal streptococci bacteria. The preliminary incubation at 35°C helps for the recovery of stressed organisms. For *Pseudomonas* sp., *Pseudomonas* agar base medium used and incubated at 24^{o}C for 48h. Developed bacterial colonies were counted and recorded as CFU per gram of the sample. Count was calculated by multiplying the average number of colonies per plate by reciprocal of the dilution and then divided by volume of culture plate. The bacterial colonies were divided into different types according to the colony shape characteristics, size, color and opacity.

2.4 Purification and identification

Depending on colonial macro morphology, three representatives of each colony type were streaked on fresh plates repeatedly until pure cultures were obtained. Identification was carried out by studying colonies characters as well as smearing of the colonies and staining with Gram's stain followed by microscopically examination for staining reaction of microorganism and demonstrating the morphology, arrangement and staining reaction of microorganism [26].

Potassium hydroxide test was carried out as confirmatory test to identify gram negative bacteria by transferring a visible amount of bacterial growth from an agar culture to a drop of 3% aqueous KOH. Presence of viscosity line with using loop indicates positive result [27]. Motility test was carried out using hanging drop method [28]. Catalase test was carried out by transferring a small amount of organism was collected from a well-isolated 18- to 24-hour colony to a drop of 3% H_2O_2. Catalase Positive reactions are evident by immediate effervescence and bubbles formation [29]. Oxidase test was carried out by transferring a small amount of organism obtained from an agar plate with a sterile swab to one drop of reagent (N, N, N, N-tetramethyl phenylenediamine dihydrochloride). Positive reactions turn the bacteria violet to purple immediately or within 10 to 30 seconds [29]. Arginine dihydrolase test was carried out by growing bacteria on the medium CFC (Cephaloridine, Fucidin and Cetrimide), which is selective for Pseudomonads. Arginine (1% w/v) and pH indicator phenol red (0.002% w/v) were added to this medium. Pink coloration of *Pseudomonas* colonies and media indicates ammonium production and alkaline drift in PH [30]. Culturing *Pseudomonas* sp. isolates on cetrimide agar shows a characteristic blue-green or yellow-green colour under UV lamp as Cetrimide enhances production of pigments such as pyocyanin and fluorescein [31].

2.5 Molecular analysis

2.5.1. DNA extraction

For DNA extraction, the isolates, which were suspected to be *Pseudomonas aeruginosa*, were inoculated into nutrient broth media and incubated for 2 days on shaker (100 rpm). Then one ml of each isolates was transferred into Eppendorf microtube. The samples were centrifuged by using Microcentrifuge (6000 rpm), supernatants were removed. The pellets were washed twice by using sterile distilled water to ensure that there is no remains of media. The pellets were suspended in 500 µL of TES extraction buffer containing 50 mg/mL proteinase K and the nucleic acids were extracted. The quality of the extracted DNA was obtained by means of electrophoresis in 0.7% agarose gels, followed by staining with Ethidium bromide. The purity of the DNA was estimated spectrophotometrically using A_{260}/A_{280} ratio, whereas the yield was obtained by measuring absorbance at 260 nm with a spectrophotometer. DNA purity was further confirmed by digestion with 5 restriction enzymes (EcoR I, Rsa I, Taq I, EcoR V, Hind III) followed by gel electrophoresis [32].

2.5.2. Inter-simple sequence repeats (ISSR) PCR primers

The set of 8 ISSR primers were of 17 nucleotides in length with two nucleotides repeating eight times and having one nucleotide at 3 as an anchor or trinucleotide repeats of more than three iterations were used.

PCR was carried out according to Inter-simple sequence repeats (ISSR) protocol. The ISSR-PCR technique permits to screen quickly a wider part of genome without prior knowledge of DNA sequence. Further, the technique of ISSR amplification is sensitive enough to differentiate closely related individuals and assess the genetic diversity in germplasm [33]. The list of ISSR gene specific primers used in present study is shown in Table [1].

Table 1. List of inter-simple sequence repeat (ISSR) primers

Primer code	Repeat motif	Primer sequences
807	$(AG)_8T$	5′ AGAGAGAGAGAGAGAGT 3′
810	$(GA)_8T$	5′ GAGAGAGAGAGAGAGAT 3′
835	$(AG)_8YC$	5′ AGAGAGAGAGAGAGAGYC 3′
841	$(GA)_8YC$	5′ GAGAGAGAGAGAGAGAYC 3′
857	$(AC)_8YG$	5′ ACACACACACACACACYG 3′
HB9N31	$(GT)_6GC$	5′ GTGTGTGTGTGTGC 3′
HB12N31	$(CAC)_3GC$	5′ CACCACCACGC 3′
HB14N31	$(GTC)_3GC$	5′ GTCGTCGTCGC 3′

2.5.3. Amplification of 16S rDNA by Polymerase Chain Reaction (PCR)

Inter simple sequence repeat (ISSR) technique is a PCR based technique which involves amplification of DNA segments between two identical microsatellite repeat regions ‘oriented in opposite direction using primers designed from microsatellite core regions. The technique uses microsatellite primers, usually 16−25bp long, of di-nucleotide, trinucleotide, tetra-nucleotide or penta-nucleotide repeats to target multiple genomic loci. The primers can be either unanchored or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences [33].

PCR amplification the ISSR markers, resolved on 1.5 % agarose gel. 100 bp ladder (L) from Vivantis Company was loaded between the PCR products of each primer. The PCR amplification using ISSR primers was formed with following conditions : Denaturation at for 95°C for 5 minutes, annealing at 45°C for 30 seconds and extension at 72°C for 30 seconds, for 35 cycles followed by7 minutes extension at 72°C. Each 10 µl PCR reaction contained 2 µl of genomic DNA, 1 µl primer (10 pomol), 10 mM dNTPs, 2 µl buffer, 0.1 taq DNA polymerase(from my Taq company). Clearly, distinctive and strong insensitive bands were only scored. The cloned fragments were separated and subjected to sequence analysis using the Big TriDye sequencing kit (ABI applied Biosystems) by the facility of macrogen, Korea.

2.5.4. Phylogenetic analysis

Sequences were blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment by MEGA X software for construction of phylogenetic tree with 500 bootstrap replication. The evolutionary distances were computed using the Maximum Composite Likelihood method [34].

2.5.5. Antibiotic susceptibility test

Kirby-Bauer disc diffusion method was adopted for antibiotic susceptibility testing. Pure colonies of the two identified *Pseudomonas aeruginosa* strains were cultured on Mueller-Hinton agar by spread plating using a sterile swab. For each strain, three replicates were conducted. Antibiotic discs were placed onto the surface of agar plates using sterile forceps. Reasonable spaces were considered among different discs. Agar plates were incubated at 37°C for 24 hrs. The zones of inhibition were measured using a meter ruler [35].

The investigated antibiotics were: - Tetracycline (10 µg), Gentamicin (10 µg), Ampicillin (10 µg), Trimethoprim/ Sulphamethoxazole (25 µg), Vancomycin (30 µg), Erythromycin (15 µg), Ciprofloxacin (30 µg), Chloramphenicol (30 µg), Amoxicillin (10 µg) and Rifampicin (5 µg).

3. RESULTS

3.1. Microbial analysis results

Results in table 2 show that total viable bacterial count was concentrated in intestine and gills with mean 205 x 10^4 $CFUg^{-1}$, 46 x 10^4 $CFUg^{-1}$ in winter and 222 x 10^4 $CFUg^{-1}$, 144 x 10^4 $CFUg^{-1}$ in summer respectively, while the lowest ones were recorded in flesh with mean 1 x 10^4 $CFUg^{-1}$ in winter and 2 x 10^4 $CFUg^{-1}$ in summer. Total coliform count was concentrated in intestine and gills with mean 170 x 10^3 $CFUg^{-1}$, 120 x 10^3 $CFUg^{-1}$ in winter and 425 x 10^3 $CFUg^{-1}$, 361 x 10^3 $CFUg^{-1}$ in summer respectively, while the lowest ones were recorded in flesh with mean 2 x 10^3 $CFUg^{-1}$ in winter and 3 x 10^3 $CFUg^{-1}$ in summer. Faecal coliform count was concentrated in intestine and gills with mean 22 x 10^2 $CFUg^{-1}$, 43 x 10^2 $CFUg^{-1}$ in winter and 68 x 10^2 $CFUg^{-1}$, 90 x 10^2 $CFUg^{-1}$ in summer respectively, while the lowest ones were recorded in flesh with mean 2 x 10^2 $CFUg^{-1}$ in winter and 2 x 10^2 $CFUg^{-1}$ in summer. There is no faecal streptococcus isolated from different fish organs in winter. However, in summer, it is isolated from intestine, gills and flesh with mean 1 $CFUg^{-1}$, 5 $CFUg^{-1}$ and 1 $CFUg^{-1}$ respectively. It was obvious that the presence of faecal coliform was affected by temperature. It was obvious that the presence of faecal coliform was affected by temperature. *P. aeruginosa* was concentrated in intestine and gills with mean 79 X 10^2 $CFUg^{-1}$, 46 X10^2 $CFUg^{-1}$ in winter and 185 x 10^2 $CFUg^{-1}$, 178 X 10^2 $CFUg^{-1}$ in summer respectively, while the lowest ones were recorded in flesh with mean 0.7 X 10^2 $CFUg^{-1}$ in winter and 4 X 10^2 $CFUg^{-1}$ in summer.

Table 2. Bacterial count means of different organs in *Oreochromis niloticus* at different seasons

Organ	Season	Mean CFU g^{-1} ± S.E.				
		TBC	TC	FC	FS	Ps
Intestine	winter	205 ±42	170 ± 3	22 ± 0.8	0	79 ± 8
	summer	222 ±5	425 ± 7	68 ± 0.7	1	185 ± 61
Gills	winter	46 ± 0.3	120 ± 5	43 ± 8	0	46 ± 44
	summer	144 ±30	361 ± 7	90 ± 5	5	178 ±16
Flesh	winter	1 ± 0.1	2 ± 0.2	2 ± 0.07	0	0.7 ± 0.01
	summer	2 ± 2	3 ± 0.3	2 ± 0.2	1± 0.3	4 ± 0.7

TBC: Total Bacterial count (CFU X 10^4/g); **TC:** Total Coliform (CFU X10^3/g); **FC:** Faecal coliform (CFU X 10^2 /g); **FS:** Faecal Streptococcus (CFU/g); ***Ps:*** *Pseudomonas* sp. (CFU X 10^2/g).

3.2. Identification of bacteria

From the CFC agar plates, 20 presumptive *Pseudomonas* cultures were isolated. They are bacilli and confirmed the negative reaction (Gram -) to Gram stain and positive reaction to KOH test. In nutrient agar, the colonies were from creamy to straw color, circular, convex, entire margin, smooth and shiny, some with pigmentation, which varied between brown and pale yellow. As shown in table (3), about 16 isolates were motile. All isolates had catalase enzyme. However, 12 isolates only had oxidase enzyme. From these isolates, 6 isolates had Arginine dihydrolase enzyme forming pink color. All of these 6 isolates had grown in cetrimide agar. However, only two isolates had given fluorescent using UV.

Table 3. Biochemical characterization of isolated bacterial isolates

Test\isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Organ	I	F	F	G	F	I	I	G	G	G	F	F	I	G	F	I	I	F	G	I
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
KOH test	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	+
Catalase reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase reaction	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-
Arginine dihydrolase growth color	P	Y	Y	Y	Y	Y	P	Y	Y	Y	P	Y	P	Y	P	Y	Y	Y	P	Y
Cetrimide agar	+	-	-	-	-	-	+	-	-	-	+	-	+	-	+	-	-	-	+	-
UV flourescene	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-

* **I**= intestine, **G**= gills, **F**= flesh

Y= yellow color, **P**= pink color.

3.3.Molecular analysis results

In PCR, the four primers 857, HB9N31, HB12N31 and HB14N31 showed positive amplification as illustrated in the gel photo. The results are shown in figure 2. By using the Big TriDye sequencing kit (ABI applied Biosystems), the isolates No. 1 and 2 were identified as two strains of *P. aeruginosa* based on the morphological, biochemical properties and 16S rRNA gene analysis (Table 4 and 5).

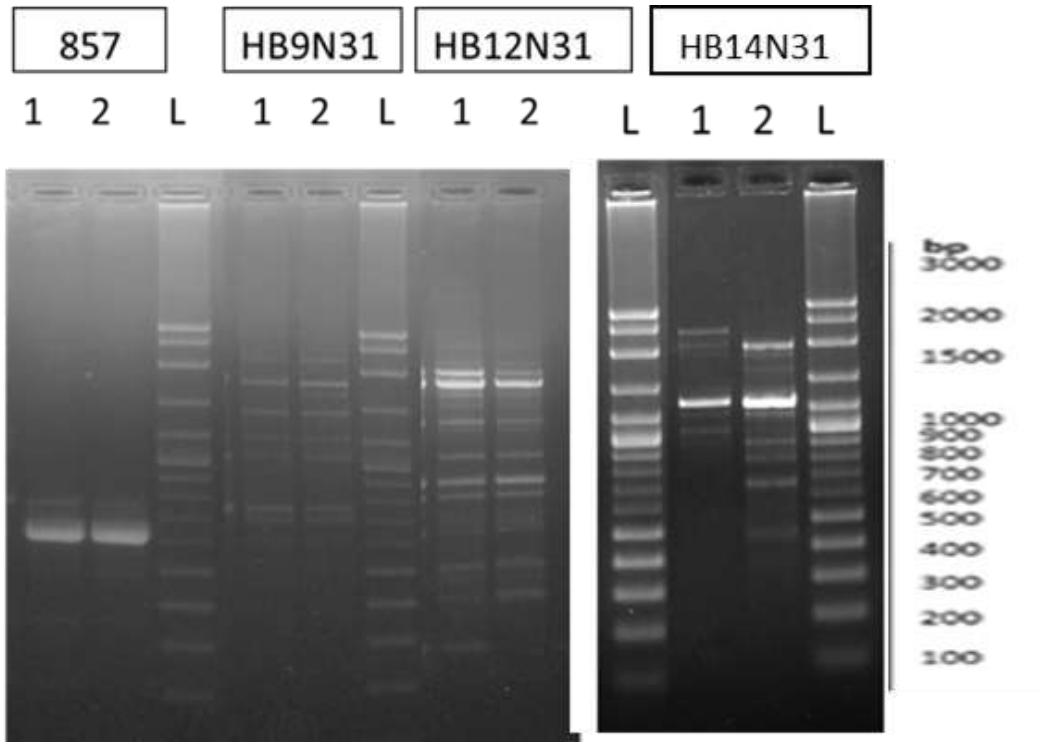

Fig.2. ISSR profiles of Agarose (1.5%) gel electrophoresis of ISSR-PCR products for 4 ISSR primers (857, HB9N31, HB12N31 and HB14N31) of isolates**. L**: 100 bp ladder; **1**:isolate 1; **2**: Isolate 2.

Table 4. The nucleotide sequence of the two bacterial isolates.	
Strain	Aligned Sequence Data
Pseudomonas aeruginosa strain PF28	CTAGGAATCTGCCTGGTAGTGGGGGATAACGTTTCGAAAGGGACGCTAATAC CGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAG ATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCTA CGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACG GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAA AGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGC ACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAATATCTTGCTGTTTTGACGTT ACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACA GAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGG TTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAA AACTGGCAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGT GAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGA CTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATA

	ACTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCG CAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAG AGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTG CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGA GCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAG ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG CCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGC CAAGCCGCGAGGTGGAGCTAATCCCACAAAACCGATCGTAGTCCGGATCGC AGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGA ATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT GGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTAC CACGGTGTGATTCATGACTGGGGTGAAGTCGTAAC
Pseudomonas aeruginosa strain C1	ATTAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACAT GCAAGTCGAGCGGATGACGGGAGCTTGCTCCTTGATTCAGCGGCGGACGGGT GAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGA ACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTT GCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTC ACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGA ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCG GATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCT GTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCG CGGTAATACAGAGGGTGCAAGCGTTAATCGGAGTTACTGGGCGTAAAGCGC GCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAA CTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCT GTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTT GGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGG GGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACA AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGC CTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTG ACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGG CACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC AAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGT ACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGT AGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAA TCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC CCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGG GAGGACGGTTACCACGGTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGT AAAT

Phylogenetic analysis of *Pseudomonas* strains (*Pseudomonas aeruginosa* strain PF28 and *Pseudomonas aeruginosa* strain C1) was conducted by using the nucleotide sequences of the genes for 16S rRNA, which has been determined by the direct sequencing of PCR-amplified fragments. Phylogenetic trees based on

these data were constructed by the neighbour-joining method using the genetic distances adjusted by the Jukes-Cantor formula for multiple substitutions as shown in figure 3.

Table5. Molecular identification of the two strains based on 16SrRNA gene analysis

isolate	genus	Per. identity	Accession number
1	*Pseudomonas aeruginosa* strain PF28	97%	MF838682.1
2	*Pseudomonas aeruginosa* strain C1	97.53%	HM560953.1

Fig.3.Phylogenetic tree depicting phylogenetic relationships of the two strains isolated from this study (*Pseudomonas aeruginosa* **strain PF28** and *Pseudomonas aeruginosa* **strain C1**) using the Maximum Composite Likelihood method. Accession numbers of sequences included in the tree. Thestudied Strains PF28 and C1 are closely related with known taxa *Pseudomonas aeruginosa* w935-H and *Pseudomonas aeruginosa* IRLM4 with similarity of 97% and 97.53 respectively

3.4. Antibiotic susceptibility test result

Antibiotic susceptibility test was carried out for two isolates which have been confirmed that they are *pseudomonas* strains by molecular identification. As shown in figure (4), the susceptibility patterns obtained revealed varying degrees of resistance and sensitivity to the antibiotics used in the screening. These profiles were determined from the interpretation of the inhibition zone diameter of these antibiotics on the organisms. For *Pseudomonas aeruginosa* strain PF28, it was sensitive only to two antibiotics; Gentamycin 10 µg and Ciprofloxacin 30 µg, while it was resistant to Tetracycline (10 µg), Ampicillin (10 µg), Trimethoprim/ Sulphamethoxazole (25 µg), Vancomycin (30 µg), Erythromycin (15 µg), Chloramphenicol (30 µg), Amoxicillin (10 µg) and Rifampicin (5 µg). For *Pseudomonas aeruginosa* strain C1 was sensitive antibiotics; Gentamycin 10 µg, Rifampicin 5 µg and Ciprofloxacin 30 µg, while it was resistant to Tetracycline (10 µg), Ampicillin (10 µg), Trimethoprim/ Sulphamethoxazole (25 µg), Vancomycin (30 µg), Erythromycin (15 µg), Chloramphenicol (30 µg) and Amoxicillin (10 µg) as shown in table (5).

Table .6. Antibiotic sensitivity test results for 2 *Pseudononas* sp. strains

Strains\Antibiotic	CN	SXT	VA	TE	AMP	E	C	CIP	RD	AML
Pseudomonas aeruginosa strain PF28	+	-	-	-	-	-	-	+	-	-
Pseudomonas aeruginosa strain C1	+	-	-	-	-	-	-	+	+	-

Anibiotics: **CN**: Gentamycim (10 µg) ; **SXT**: Trimethoprim/ Sulphamethoxazole (25 µg); **VA**: Vancomycin (30 µg); **TE**: Tetracycline (10 µg) ; **AMP**: Ampicillin (10 µg) ; **E**: Erythromycin (15µg); **C**: Chloramphenicol (30 µg) ; **CIP**: Ciprofolxacin (30 µg); **RD**: Rifampicin (5 µg) ; **AML**: Amoxicillin (10 µg)

(+) : Sensitive to antibiotic**; (-):**Resistant to antibiotic.

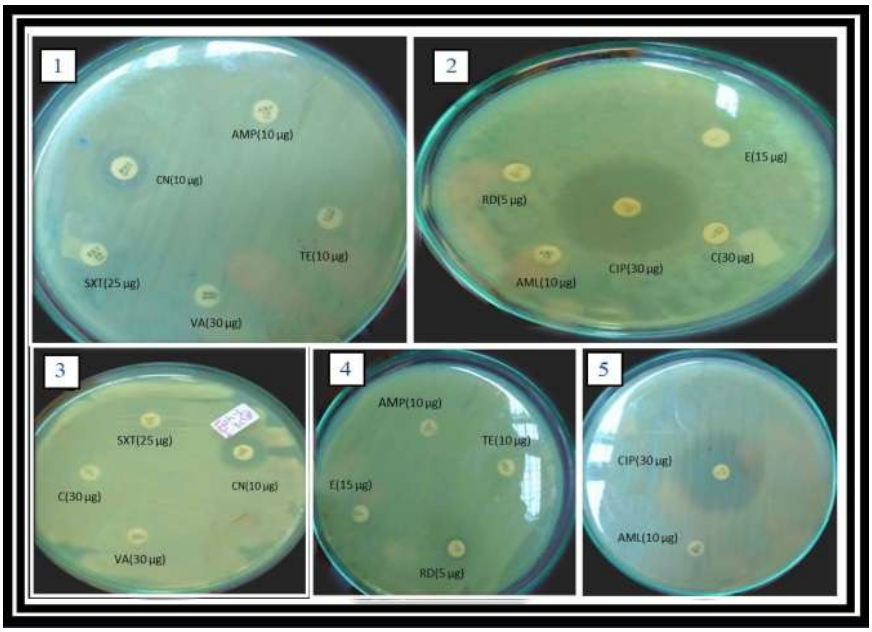

Fig. 4. Results of antibiotic susceptibility test for both isolates: Plate 1, 2 for *Pseudomonas aeruginosa* strain PF28 and plate 3, 4, 5 for *Pseudomonas aeruginosa* strain C1.The names of the 10 selected antibiotics were written on each plate

4. DISCUSSION AND CONCLUSION

Nile tilapia, *Oreochromis niloticus* L. is the most prevalent species of the bony fish in Africa including Egypt and an important fish in the ecology of tropical and sub-tropical region [36]. This is attributed to many positive qualities including tolerance to poor water quality, wide range of food, plasticity in growth, firm flesh, great taste and its efficiency to convert organic and domestic wastes into high quality protein [37, 38]. Besides, *O. niloticus* is characterized by short generation time due to extended breeding seasons and its

reproductive biology [39] and therefore spawning season expanded from March to September [11]. Fish catches in Lake Manzala have been dominated by tilapia species over the past 70 years. The predominance of the tilapia fishery in the lake is due to their high tolerance for marginal environmental conditions in terms of oxygen concentrations and high nutrient loadings [40].

The contamination of water bodies in the Egyptian Nile Delta region including Lake Manzala is exposed to release of enormous amounts of domestic sewage as well as agricultural and industrial effluents [41]. The bacterial load of fish is a reflection of the water status in which they were caught and therefore, fish can acquire pathogenic microorganisms from the natural aquatic environment [42]. It has been reported that all the fish samples collected from Lake Manzala were contaminated on surface and internally with exceptionally high amounts of bacteria at gill and intestine confirming that lake fish is profoundly polluted and dangerous for human health [43].

The current study revealed that the total bacterial counts mean (TBC) of different studied organs of tilapia fish ranged from 1×10^4 $CFUg^{-1}$ to 222×10^4 $CFUg^{-1}$. These results vary with the results of recently previous study on tilapia fish taken from Manzala Lake of Damietta Governorate which concluded that TBC mean for tilapia fish ranged from 0 to 4400×10^3 $CFUg^{-1}$ in different seasons [43]. This contrast might ascribe to the differences in space and time of the bacterial counts and the chemical parameters [44].

Egyptian General Authority of Standardization and Quality Control considered 10^6 CFU total bacterial counts per gram as a maximum permitted limit for fish [42, 45]. This means that the result of TBC recorded in this study is higher than recommendation of Egyptian General Authority of Standardization and Quality Control.

Regarding fish intestine , the total bacterial counts mean (TBC) of ranged from 204.5×10^4 CFU g^{-1} in winter to 222×10^4 CFU g^{-1} in summer. Moreover, another author recorded higher values of TBC of tilapia intestine organ which ranged from 1×10^7 CFU g^{-1} to 3×10^7 CFU g^{-1} in winter and from 1×10^5 CFU g^{-1} to 1×10^6 CFU g^{-1} in summer [46]. Relatively different results were obtained by another where TBC of tilapia intestine organ was 10×10^3 CFU g^{-1} in winter and 6000×10^3 CFU g^{-1} in summer [43]. However, similar results was recorded from the intestine of tilapia fish collected from new inlet of El-Gamil where the TBC was 10×10^4 $CFUg^{-1}$ [15].

Comparing the total bacterial counts mean (TBC) of fish gills with other studies in the same lake, it is obvious that [46] recorded lower TBC of tilapia gills organ was 3×10^4 CFU g^{-1} in winter and ranged from 3×10^2 CFU g^{-1} to 4×10^3 CFU g^{-1} in summer than those in the present study where the values of TBC ranged from 46×10^4 CFU g^{-1} in winter to 144×10^4 CFU g^{-1} in summer. Similar higher values of TBC were recorded in fish flesh where TBC of fish flesh in this study ranged from 1×10^4 CFU g^{-1} in winter to 2×10^4 CFU g^{-1} in summer than other studies in the same lake [43, 46].

Total coliform (TC) is an indicator of sewage contamination [47]. The results show that TC varies greatly between different organs during summer and winter where the values ranged from 2×10^3 $CFUg^{-1}$ in flesh during winter to 425×10^3 $CFUg^{-1}$ in intestine during summer. These results much with previous studies that the total bacterial load was observed in gills and intestine comparing with other organs [15]; Besides, Al-Harbi [48] confirmed the wide variations in distribution pattern and types of bacteria in different seasons.

Arannilewa *et al*. found that the mean of total coliforms count in fish was between 3×10^3 $CFUg^{-1}$ - 8×10^6 $CFUg^{-1}$ [49]. These results were quite in agreement with results recorded by this study where TC ranges from 2×10^3 $CFUg^{-1}$ to 425×10^3 $CFUg^{-1}$. However, The result differ with Abdelhamid *et al*., [15] who concluded that TC of Tilapia fish collected from the new inlet of El-Gamil ranged between 0 and 50×10^3 $CFUg^{-1}$ in winter and summer, respectively, whereas The mean of TBC and TC in fish collected from Lake Manzala is higher, This might be due to pollution of Lake Manzala.

Regarding Fecal coliforms in fish, they outline the level of pollution of their environment scince they are not the normal flora of bacteria in fish [50] and are the most common bacteria of thermo-tolerant coliforms

[51]. Faecal coliform in fish .In the current study, all tested fish samples were contaminated by fecal coliforms bacteria (FCB).

The results also revealed that the mean of faecal coliform count of Tilapia intestine was 22 x 10^2 $CFUg^{-1}$ in winter and 68 x 10^2 $CFUg^{-1}$ in summer. This result is lower than those recorded by another authors with mean 3 × 10^4 $CFUg^{-1}$ [54]. However, fecal coliform count mean of fish gills was 43 x 10^2 $CFUg^{-1}$ in winter and 90 x 10^2 $CFUg^{-1}$ in summer. This result complies with results of previous study with mean 5 × 10^3 $CFUg^{-1}$ [54]. In the current study, fecal coliform count mean of fish flesh was 2 x 10^2 $CFUg^{-1}$ in winter and 2 x 10^2 $CFUg^{-1}$ in summer. This result is relatively low with results of another author with mean 0.5 × 10^3 $CFUg^{-1}$ [54].

FAO reported that Fish of good quality should have counts total coliforms and faecal coliforms should not exceed 100 $CFUg^{-1}$ and 10 $CFUg^{-1}$ respectively [50, 52]. The results of the current study showed range higher than FAO recommendation. The presence of coliforms above the acceptable limit in fish considered a risk to the consumers because coliforms grow rapidly at ambient temperatures and spoil the fish in a short period of time [53].

Faecal streptococci (FS) are bacteria always present in the intestinal tracts of warm-blooded animals [54]. The presence of FS in fish organs indicates the animal activity in the Lake. In the current study there is no indication of FS presence in all organs of Tilapia fish in winter, however, in summer, there is low count ranged from 1 $CFUg^{-1}$ in intestine to 5 $CFUg^{-1}$ in gills. It is obvious that fecal streptococcus activity was higher than in summer than in winter. It agrees with a previous study which reported that FS affected by temperature [55]. The results of the current study contrasted with another previous study which recorded that FS count of tiapia fish collected from Matarya ranged from 3576 $CFUg^{-1}$ in flesh to 10143 $CFUg^{-1}$ in intestine [56]. Thus Lake Manzala fish (tilapia) might be highly polluted and dangerous for human health [57].

Pseudomonas sp. is most frequently associated with human infection; however, it naturally exists in the environment [58]. In this study, *Pseudomonas* spp. reflected very high distribution in organs; gills and intestine. *Pseudomonas* sp. counts mean of Tilapia intestine was 79 x 10^2 $CFUg^{-1}$ in winter and 185 x 10^2 $CFUg^{-1}$ in summer. In gills of tilapia, the counts mean in winter and summer was 46 x 10^2 $CFUg^{-1}$ and 178 x 10^2 $CFUg^{-1}$ respectively, in flesh, counts mean was 70 $CFUg^{-1}$ in winter and 400 $CFUg^{-1}$ in summer. These results contrasted with another study in Saudi Arabia on Tilapia which recorded that there was complete absence of *Pseudomonas* in summer, while higher activity was observed in winter [48]. Bacterial pathogens can survive and exploit their hosts' cellular processes to mediate their effects intracellularly or extracellularly [5].

Bacteriological examination revealed presence of *Pseudomonas* bacteria. Morphologically, the suspected isolates were subjected to Morpho-biochemical test and two isolates of the total bacterial cultutres were identified as *Pseudomonas aeruginosa* as they give yellow-green fluorescence color on Cetrimide agar medium.

The use of 16S rRNA gene sequences to study bacterial taxonomy and phylogeny has been by far the most common genetic marker as the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes [58].The Inter simple sequence repeat (ISSR) primers that anneal to a simple repeat of various length and at non- repetitive motifs at 3 and 5 end were attempt for PCR amplification of *Pseudomonas aeruginosa*.

Of the 8 primers for repeat sequences utilized in this study, only four primers $(AC)_8YG$,$(GT)_6GC$,$(CAC)_3GC$ and $(GTC)_3GC$ produce amplified bands of 1000 bp which is helpful for identification .The remaining four ISSR primers did not exhibit any amplification. Bacteria can be identified by nucleotide sequence analysis of the PCR product followed by comparison of this sequence with known sequences stored in a database. Two strains was identified as *Pseudomonas aeruginosa* strain PF28 and *Pseudomonas aeruginosa* strain C1 with similarity 97% and 97.53% respectively. The results therefore,

confirmed the ability of primers, based on simple sequence repeat motif, to produce a fragment that is useful as a group genetic marker in the different two strains of *Pseudomonas aeruginosa*.

Antibiotic resistant bacteria isolated from tilapia fish catched from Lake Manzala were investigated. *Pseudomonas aeruginosa* is a notoriously difficult organism to control with antibiotics [59]. *Pseudomonas aeruginosa* is a notoriously difficult organism to control with antibiotics or disinfectants [59]. Its general resistance is due to a combination of factors: It is intrinsically resistant to antimicrobial agents due to low permeability of its cell wall, genetic capacity to express a wide repertoire of resistance mechanisms, mutation in chromosomal genes which regulate resistance genes and acquiring additional resistance genes from other organisms via plasmids, transposons and bacteriophages. There are three basic mechanisms by which organisms resist the action of antimicrobial agents: restricted uptake and efflux; drug inactivation and changes in targets [60]. Given this high level of natural resistance, mutational resistance to most classes of antibiotics can readily arise.

Investigation of susceptibility of the two identified isolates of *Pseudomonas aeruginosa* to 10 different antibiotics revealed that that the two strains have Multi drug resistant (MDR) to seven antibiotics. Multi drug resistance to strains is defined as being resistant to four or more antimicrobial agents [61] but sometimes as low as two antibiotics from different classes [62]. It may be due the fact that the proportion of bacteria resistant to ampicillin, tetracycline and nitrofurantion was observed among bacteria isolated from fish. The simultaneous resistance of isolates to ß - lactone and chloramphenicol may be due to the dissemination of antibiotic resistance plasmids in the marine environment [63]. Moreover, the selective pressure produced by the intensive disposal of Pharmaceutical wastes into water leading to the emergence and maintenance of Antibiotic resistant bacteria in coastal water has been documented [64].

Fish contamination with antibiotic-resistant bacteria can be a major threat to public health, as it can be transferred to other bacteria of human clinical significance. The choices of antibiotic for the treatment of common infectious diseases in humans are becoming increasingly limited, expensive and ineffective due to the emergence of antibiotic resistant bacteria [57].

In this respect it is possible that antibiotic resistance faecal bacteria of domestic sewage discharged into the lake might transfer their antibiotic resistant determinant to indigenous flora of fish, provoking their spread and prevalence in marine environment [25].

In this context , it was proposed that entry of antibiotics and of faecal antibiotic resistant bacteria into the lake might be expanding the numbers of resistant bacteria in commercial fish with possibilities of transfer of their resistance determinants to human pathogenic bacteria, Additionally, retuning of resistant enteric bacteria to human fish consumers might occur, demanding further epidemiological and molecular investigation to evaluate the presence of genetically mobile antibiotic resistant genes in the human and animal food chain [65].

There are several reports regarding antibiotic resistance in fish pathogens [65]. The resistance is an indication of the presence of β-Lactamases which are common in bacterial pathogens found in polluted water environment and the Egyptian environment [56]. In a previous study, it has been reported that *Pseudomonas aeruginosa* isolated from different organs of fishes of Lake Manzala, showed high frequency of multi-drug resistance to many antibiotics, particularly, penicillin, ampicillin and chloramphenicol, which is genetically due to harboring plasmid DNA with different sizes [67]. The high rate of resistance to the antibiotics studied could be resulted from their prevailing abuse in the area under study. This high resistance rate, which was observed in this study, is coordinated with incidences of increased antibiotic resistance reported among Gram-negative bacilli *Pseudomonas aeruginosa* in other parts of the world [68]. This supports the significance of *Pseudomonas aeruginosa* in fish in Lake Manzala, especially in the most important sites where fishing and different human activities, as causal agent of many diseases in the populations in the Lake Manzala area, where sea food is the main source of food. This represents a potential hazard to human health especially those who are on immunosuppressive drugs.

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ACKNOWLEDGEMENT

Thanks greatly to Dr. Mona El-Bous; the head of Botany Department, Faculty of Science, Port-Said University, for her help and support in this paper.