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Genotypic characterization of some dermotropic and systemic bacterial pathogens affecting two commercial Red Sea fishes

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ABSTRACT

The genotypic characterization of some bacterial pathogens that were incriminated in disease outbreaks among Haffara seabream "*Rhabdosargus haffara*" and marbled spinefoot "*Siganus rivulatus*" was investigated in full through the current study. A total of 250 fish samples (125 of each species) were collected along the Red Sea coasts of Hurghada City, Egypt. Fish samples were inspected for clinical signs, post-mortem changes, and bacteriological examination. The investigated fishes displayed septicemic signs and external skin lesions characteristic for streptococcosis and tenacibaculosis. *Enterococcus* spp., *Streptococcus* spp., and *Flavobacterium* spp. were isolated from moribund fishes using selective media. These isolates were phenotypically and genetically identified and characterized. The identities of bacterial isolates were confirmed as *Enterococcus fecalis*, *Streptococcus iniae*, *Enterobacter cloacae*, and *Tenacibaculum maritimum* based on sequencing the 16S rRNA gene. *Enterococcus fecalis* was the most common pathogen isolated from *S. rivulatus* and *R. haffara* and accounted for 46.8% and 47.8% of the total isolates, respectively. Accordingly, the present study proved that *Enterocoocus* spp., *Streptococcus* spp., and *T. maritimum* are important pathogenic bacteria incriminated in wild fish outbreaks in the Red Sea in Egypt. These findings proved the importance of regular and permanent bacteriological examination of wild fish to overcome fish mortalities, which lead to economic losses. Finally, sequencing and phylogenetic relationship techniques proved their usefulness as an ideal assay to develop a reliable, accurate, and rapid detection method for bacterial pathogens in the aquatic environment.

INTRODUCTION

The Red Sea is a worldwide hotspot of marine biodiversity with diverse commercial fish species. However, studies on the microbiological characteristic in terms of pathogenic bacteria are greatly understated. The Red Sea is regarded as the entrance of the Indian Ocean between Asia and Africa **(Rasul *et al*., 2015).** The Egyptian coasts of

the Red Sea are nearly 1080 km and have a unique geographical biochemical characteristic **(Maiyza *et al*., 2020).** Fisheries of the Egyptian Red Sea have economic importance by securing seafood items **(El-Sheshtawy *et al*., 2014),** and its attractive touristic sites provide a source of income for Egyptians through tourism activities. In addition, the Red Sea has several oil companies, and oil drilling facilities that are distributed onshore and offshore of its coasts **(Hanna, 1995).** The Red Sea coasts have been severely exposed to various pollution activities attributed to tourism, industrialization, human activities, extensive fishing, oil processing, and shipping pollution **(Mohamed *et al*., 2011).** Therefore, the Red Sea is the most hydrocarbon polluted water body worldwide **(El-Sheshtawy *et al*., 2014).** The alterations of chemical, biological, and physical parameters of the Red sea aquatic environment beyond acceptable limits could act as stress inducers and compromise the immunity of fish leading to bacterial disease outbreaks.

The marbled spinefoot "*Siganus rivulatus*" and the Haffara seabream "*Rhabdosargus haffara*" are among the most important commercial marine Red Sea fishes in Egypt **(GAFRD, 2018).** *Siganus rivulatus* is locally known as sigan and is belonging to the family Siganidae. This species is herbivorous teleost, is widely distributed in the Red Sea ecosystem, and inhabits mainly shallow water, coral reefs, seaweed, and lagoons. This fish is regarded as Lessepsian migrants and inhabited the Mediterranean Sea (**Gabr *et al*., 2018).** According to **Abdelhak *et al.* (2020),** the average catch of sigan was 500 tons from the Egyptian Red Sea in 2018. On the other hand, *R. haffara* is locally known as haffara and is belonging to the family Sparidae **(Abdelhak *et al*., 2020; Osman *et al*., 2020).** This species is a carnivorous teleost feeding on crustaceans and benthic mollusks and is a protandrous hermaphrodite **(Lin *et al*., 2021).** This fish species is demersal teleost and inhabits along the shore from shallow water to deeper water **(Mehanna *et al*., 2016).** Both fish species have high commercial values attracting the great interest of consumers in Egypt.

Mustafa *et al.* (2014) proposed that anthropogenic pollution exerts elusive and adverse impacts on the health status of marine fishes within the Red sea ecosystem. Such adverse effects may comprise heavy metal pollutants and bacterial outbreaks **(Mustafa *et al*., 2014; 2016).** Previous studies proved that fecal Enterococci and Streptococci are used as indicators for water pollution due to anthropogenic activities on the Red Sea coasts of Egypt **(El-Sheshtawy *et al*., 2014; Mahmoud *et al*., 2017).** *Streptococcus iniae*, *Enterococcus faecalis*, *Enterobacter cloacae*, and *Tenacibaculum maritimum* get a worldwide interest among fish pathologists and microbiologists **(Austin and Austin, 2016).** Streptococcosis is a worldwide aquatic animal disease that presents an actual danger for both aquatic species and human consumption. *Streptococcus iniae* is a fatal bacterial pathogen linked with severe outbreaks in wild and farmed fish **(Sun *et al*., 2013).** *S. iniae* has been isolated from a wide host range and infects at least 27 fish species, such as tilapia, Japanese flounder, rainbow trout, channel catfish, sea bass, and barramundi. The *S. iniae* infections are predisposed by numerous environmental factors and stressors that eventually could be ended with heavy mortality in fish **(Saleh *et al*., 2019).**

Enterococcus faecalis has appeared as emerged fish pathogens that cause severe economic losses among farmed and wild fish. This pathogen has been incriminated in several outbreaks and streptococcal infection in marine and freshwater fish in Egypt.

Enterococcus faecalis is the major cause of nosocomial disease in humans **(Abdelsalam *et al*., 2021).** The antibiotic-resistant strains of *E. faecalis* are widely detected in marine ecosystems, suggesting that these isolates might be virulent strains and have major public health concerns worldwide **(Di Cesare *et al*., 2012).** Tenacibaculosis is a deadly fish disease caused by *Tenacibaculum maritimum* that belongs to the family Flavobacteriaceae. This pathogen is an essential component of bacterial marine environments and is also responsible for severe threats to wild and farmed marine fish species **(Bridel *et al*., 2020).** *Tenacibaculum maritimum* infected directly the fish body surfaces triggering lesions such as eroded mouth, necrosis, ulcer, tail-rot, and frayed fins. The abraded skin allows the entrance of secondary bacterial infections, thus *T. maritimum* is frequently isolated from mixed infections **(Habib *et al*., 2014).**

On the other hand, an *Enterobacter cloaca* belongs taxonomically to the family Enterobacteriaceae **(Thillai Sekar *et al*., 2008).** It is widely isolated from human and animal feces, soil, and contaminated water. *E. cloacae* has been associated with mortalities among mullet "*Mugil cephalus*", freshwater catfish "*Pangasianodon hypophthalmus*", and common carp "*Cyprinus carpio*" fishes **(Thillai Sekar *et al*., 2008; Kumar *et al*., 2013; Al-Niaeem *et al*., 2021).** Moreover, E. cloacae has an important zoonotic significance by causing severe human infection **(Kumar *et al*., 2013).** Consequently, this pathogenic bacterium is considered a possible hazard to fish abundance in the Red Sea. However, there is a scarcity of studies investigating the occurrence of streptococcus, enterobacter, and flavobacterium infections among *R. haffara* and *S. rivulatus* inhabiting the coast of Hurghada city. Therefore, this study aimed to investigate the genotypic characterization of some dermotropic and systemic bacterial infections among marine fishes collected from the Red sea coasts of Hurghada City, Egypt. Moreover, the phenotypic characterization of these bacterial isolates was completed using different traditional bacteriological assays.

MATERIALS AND METHODS

Fish specimens

A total of 250 fish samples were collected from Hurghada city coasts, Egypt. The fish samples included two fish species; Haffara seabream "*Rhabdosargus haffara*" (n = 125) and the marbled spinefoot "*Siganus rivulatus*" (n = 125). The collected fishes were transported using an insulated ice-box to the National Institute of Oceanography and Fisheries (N.I.O.F.) in Hurghada City, for identification and clinical examination. All national and institutional regulations for the use and care of fish were monitored.

Clinical and post-mortem examination

The total length and weight were measured from each specimen. The collected *R. haffara* and *S. rivulatus* were clinically examined for external and gross lesions according to **Eissa (2016)** and **Abdelsalam *et al*. (2016, 2020).** The investigated fishes were then carefully dissected for post-mortem examination according to **Austin and Austin (2016).**

Isolation of the pathogenic bacteria

Loops from kidney, liver, spleen, and brain of moribund fishes were aseptically taken and streaked onto tryptic soya agar (TSA; Oxoid, Hampshire, UK, supplemented

with 3% NaCl), and brain heart infusion agar (BHI; Oxoid, supplemented with 3% NaCl), and then incubated aerobically at 30°C for 24 hr. For bacterial purification, single representative colonies were taken and streaked onto TSA and BHI agar, Kenner Fecal Agar (Sigma), marine agar, MacConkey agar, and 5% sheep blood agar (Oxoid). Plates were incubated at 25°C for 72 hrs. Gram stain, oxidase, catalase, and hemolysis tests were routinely used as preliminary traditional microbiological assays. Finally, the identification of the retrieved bacterial isolates was performed by using the automated Vitek 2 compact device version 07.01 (bio-Merieux), API 20E, and API 20 strep kits (bio-Mérieux), following the instruction of the manufacturer.

Genotypic identification

The extraction of genomic DNA was performed using Qiagen DNA extraction kit (Qiagen, Hilden, Germany) according to the guidelines of the manufacturer. The 16S rRNA gene was amplified using the following primer pairs (16S-F: 5'-AGAGTTTGATCCTGGCTCAG-3') and (16S-R: 5'-GGTTACCTTGTTACGACTT-3'), according to the procedure designated by **Weisburg *et al*. (1991).** PCR reaction was performed in a total volume of 25 µl, that has consisted of 12.5 µl of HotStarTaq Master Mix (Qiagen), 5 µl genomic DNA, 1.0 µl 16S-F primer, 1.0 µl 16S-R primer, and 5.5 µl of nuclease-free water. PCR cycle started with the initial denaturation step at 94°C/6 min; followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50 °C for 30 seconds, then extension at 72°C for 45 seconds; and ended with a final extension at 72°C for 10 min. The purification of PCR products was performed using the QIAquick PCR purification kit (Qiagen). The electrophoresis of amplicons was performed in 2% agarose that was dissolved in Tris-EDTA buffer accompanied with ethidium bromide (0.5 µg/ml). Gels were visualized under UV transilluminator (Winpact Scientific, USA). PCR products were directly submitted to Sigma Scientific Services Laboratory (Cairo, Egypt) to be sequenced by ABI 3730xl DNA sequencer (Applied Biosystems™, Foster City, CA, USA) in both directions.

BioEdit program was used to check and edit the obtained sequences **(Hall, 1999).** The sequences were aligned against other sequences using BLASTN search (NCBI). The edited sequences were assembled and submitted to the database of GenBank for issuing the accession numbers. The principle of bacterial identification based on 16S rRNA sequence is mainly dependent upon the 16S rRNA similarity index as described by **Drancourt *et al.* (2000).** Confirming the species identity of the bacterial isolate was achieved at ≥99% similarity score of the 16S rRNA to the related sequences in GenBank.

The phylogenetic analysis was performed by using MEGA X **(Kumar *et al*., 2018).** The following features were used: Neighbor-Joining method with 1000 bootstrap replicates, rate of variation among sites: uniform, and pattern among lineages: homogeneous, and substitutions: transversions and transitions.

Antimicrobial susceptibility

The antibiotics sensitivity was performed using the disc diffusion method on Muller–Hinton agar (Oxoid) as described by **(Abdelsalam *et al*., 2017; EI-Jakee *et al*., 2020)** using the following antimicrobial discs: ampicillin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), cefotaxime (30 µg), doxycycline (30 µg), erythromycin (15 µg), florfenicol (30 µg), oxytetracycline (30 µg), polymixin (30 µg), streptomycin (10 µg), norfloxacin (10 µg), and sulfamethoxazole–trimethoprim (25 µg).

The inhibition zones were measured and interpreted according to the Clinical Laboratory Standard Institute Guidelines, **CLSI (2014),** and then the bacterial isolates were defined as resistant or susceptible to the tested antibacterial agents.

RESULTS

Clinical findings

The clinical signs of moribund *S. rivulatus* revealed external hemorrhages, skin darkness, scale detachments, skin erosions, fin rot, ulcers, ascites, and eye opacity. Internally, moribund fish showed different septicemia lesions such as hemorrhagic liver and kidney, splenomegaly, and hepatomegaly (Fig. 1).

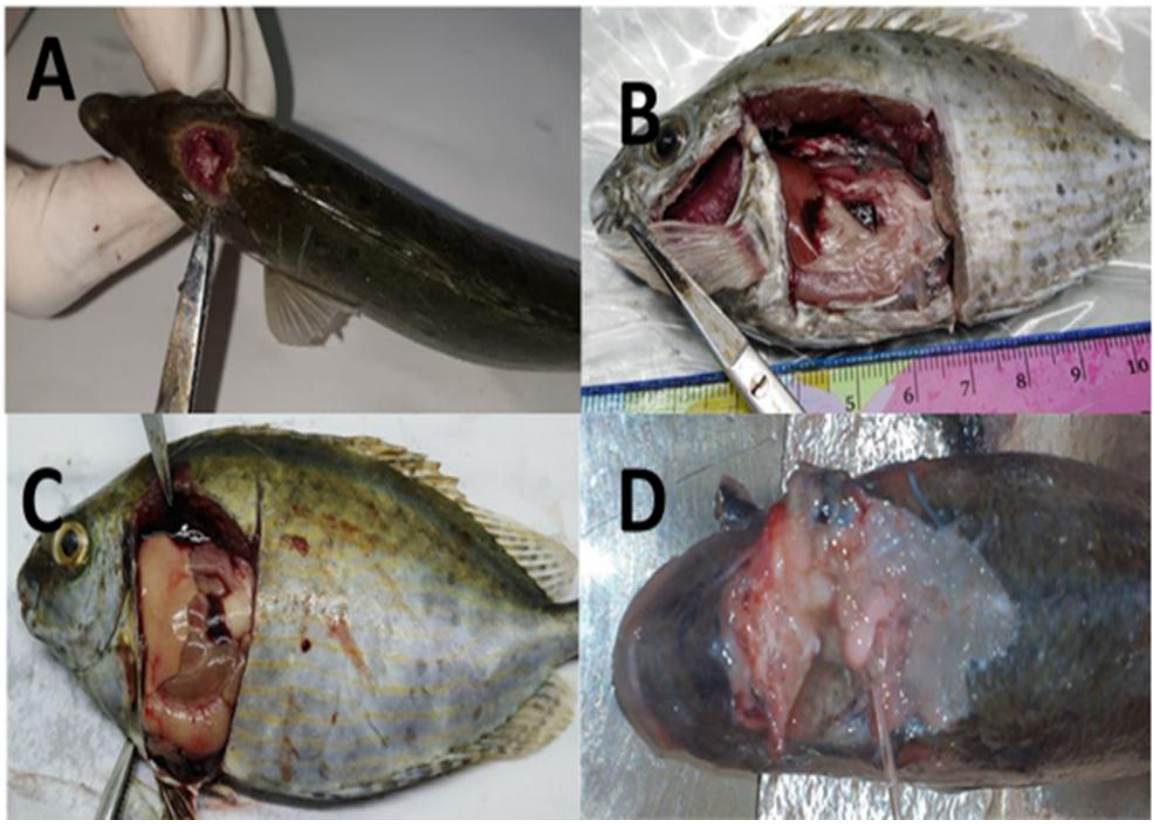

Fig. 1: A) Moribund *S. rivulatus* showing ulcer in the head region. **B)** Hemorrhagic liver and kidney. **C)** Pale liver and severe congestion in the kidney. **D)** Severe congestion in the brain.

On the other hand, the clinical signs of moribund *R. haffara* demonstrated skin darkness, external hemorrhages, erosions, and skin ulcers (Fig. 2). Internally, dissected fish showed hemorrhages in the internal organs such as the kidney, liver, and spleen. In addition, moribund fishes revealed gills congestions, and paleness of the liver and kidney.

Fig. 2: A) Moribund *R. haffara* showing congestion and hemorrhages in kidney and liver. **B)** Diseased fish revealed pale liver and kidney.

Microbiological examination

Different pathogenic bacteria were isolated and identified from moribund marine fishes using the traditional and molecular procedures. *Streptococcus iniae* was the first identified bacterium that appeared as non-pigmented colonies with 1 mm in diameter on BHIA and as β hemolytic colonies with 1-2 mm on 5% sheep blood agar. *Streptococcus iniae* isolates were fermentative and Gram-positive cocci arranged in pairs and chains. They are catalase and oxidase-negative. They cannot grow at 10 or 45°C or in 40% bile or 6.5% (w/v) sodium chloride, but they grow at 37°C and pH 9.6. Acid is produced from N-acetyl-glucosamine, aesculin, arbutin, cellobiose, D-fructose, gentiobiose, D-glucose, glycogen, maltose, mannitol, D-mannose, melezitose, ribose, salicin, starch, and trehalose. They produced alkaline phosphatase, arginine dihydrolase, ß-glucuronidase, leucine arylamidase, and pyrrolidinyl arylamidase, but not ß-galactosidase. The Voges Proskauer reaction and nitrate reduction are negative.

Enterococcus faecalis was the second identified bacterium from both fishes and appeared as yellow colonies with 1-2 mm in diameter on TSA, and as brownish colonies on bile salt agar due to bile hydrolysis. *E. faecalis* isolates were facultative anaerobic, Gram-positive cocci arranged in chains. They were negative for catalase and oxidase, indole, aesculin, sodium hippurate hydrolysis, and Voges Proskauer reaction, but they were positive for DNA, gelatin hydrolysis, 1% methylene blue reduction, nitrate reduction, hemolysis, bile esculin test, potassium tellurite, tetrazolium reduction, D-sorbitol and glycerol fermentation, and citrate utilization.

Tenacibaculum maritimum was the third identified bacterial isolates that appeared as pale yellowish and rhizoid colonies. They are Gram-negative, aerobic long and thin rod-shaped bacteria, and motile by gliding movement. They were positive for oxidase, catalase, Voges Proskauer reaction, and Congo red absorption, but they could not produce flexirubin type pigments.

Enterobacter cloacae was the fourth identified bacterial isolates that appeared as pink, mucoid, round, raised, and regular in MacConkey agar. The biochemical tests revealed that they were Gram-negative motile rod-shaped bacteria. They are oxidase negative but catalase positive. They could ferment glucose, lactose, and sucrose. They were negative for H2S production. They were positive for arginine dihydrolase, ornithine decarboxylase, and triple sugar iron but negative for urease and lysine decarboxylase.

Molecular identification

The identity of the pathogenic bacteria was confirmed by sequencing the 16S rRNA gene. The alignment of 16S rRNA sequences revealed that two Gram-positive bacteria were identified as *S. iniae* and *E. faecalis*. They were submitted to the database of GenBank under the accession numbers OK559624 and MW508512, respectively. The accession no. "MW508512" was 1427-bp and showed 99.72% 16S rRNA homology with *E. faecalis* (KR858855.1); 99.71% 16S rRNA similarity to the accession number of *E. faecalis* (MT421815.1, MF354866.1, EU168400.1, and MG694615.1); and 99.51% 16S rRNA similarity to the accession number of *E. faecalis* (CP028724.1, CP033787.1, LR962732.1, and MT356184.1). While the GenBank accession no. "OK559624" was 1482-bp and showed 99.66% 16S rRNA similarity to the accession number of *S. iniae* (CP032401.1, LC378581.1, and CP017952.1) and showed 99.59% similarity to the accession numbers of *S. iniae* (KF815728.1 and CP005941.1), and 99.53% similarity to the accession numbers of *S. iniae* (MZ366293.1, MN194173.1, HM053435.1, and CP024843.1).

On the other hand, the 16S rRNA sequences from two Gram-negative isolates were identified as *T. maritimum* and *E. cloacae* and their accession numbers were MW508513 and MW509417, respectively. The GenBank accession no. "MW508513" was 1417-bp and showed 99.79% similarity to the accession numbers of *T. maritimum* (NR_113825.1, AB078057.1, and KT270382.1), and 99.72% similarity to the accession numbers of *T. maritimum* (AB681030.1, LT634361.1, and MW690171.1). However, the GenBank accession no. "MW509417" was 1393-bp and showed 99.78% similarity to the accession numbers of *E. cloacae* (CP020528.1, CP033466.1, KX156583.1, and MF144477.1).

The phylogenetic tree displayed two main clades. The first linage was separated into two subclades. The first one involved *E. faecalis* and *S. iniae* isolates that separated into two branches forming a distinct phylogenetic subclade with 100% bootstrap value. The two isolates of *E. faecalis* and *S. iniae* are embedded among other related bacterial isolates and separated from *E. cloacae* isolates. The second subclade is comprised of the current isolate of *E. cloacae* that is embedded with other *Enterobacter* spp. isolates with strongly supported by 98% bootstrap value. The second clade included *T. maritimum* that separated from *F. columnare* and form a monophyletic group with 100% bootstrap value (Fig 3).

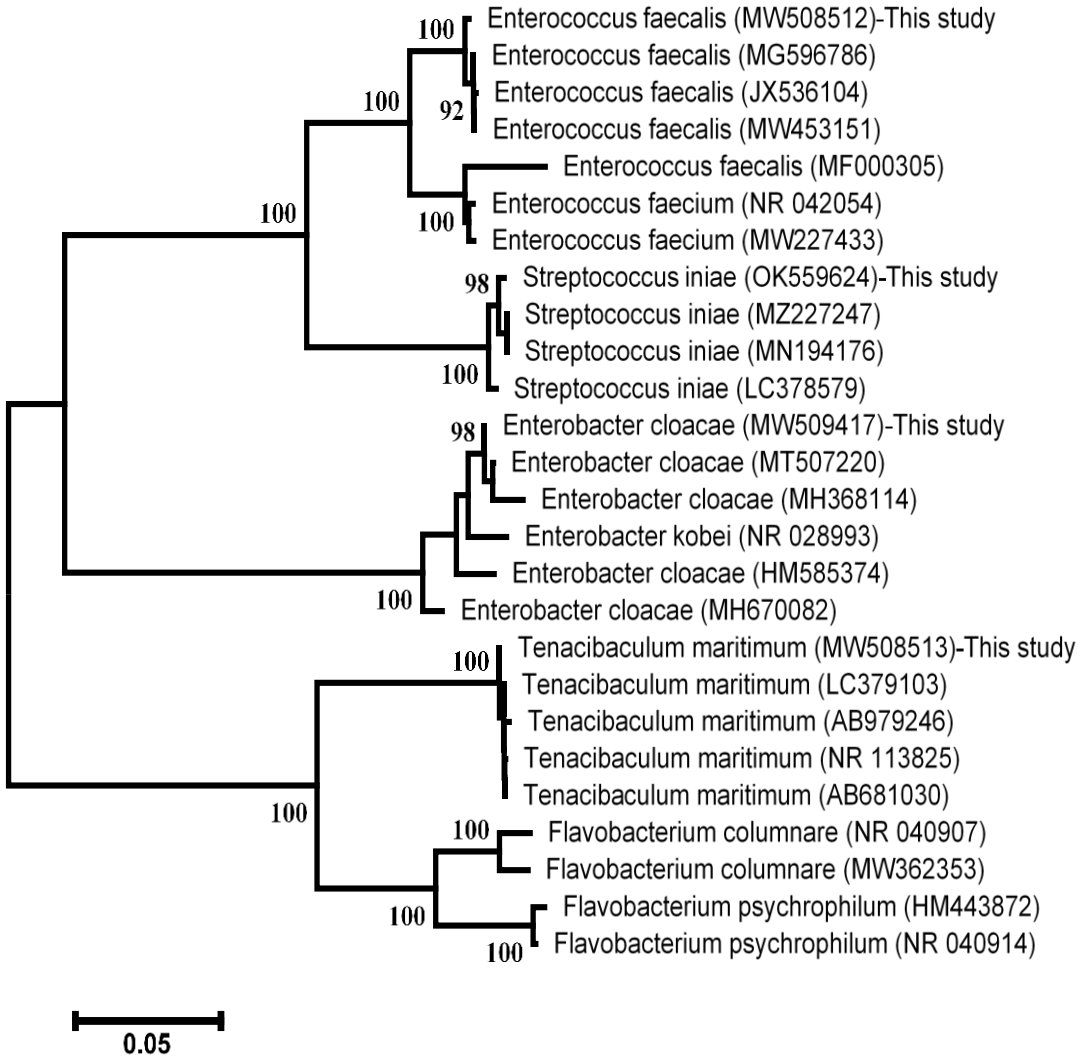

Fig. 3: The neighbor-joining phylogenetic tree is based on the comparative sequences of 16S rRNA genes of retrieved pathogenic bacteria from moribund fishes.

Incidence of pathogenic bacteria

A total of 47 pathogenic bacterial isolates were isolated from diseased *S. rivulatus. Enterococcus faecalis* was the most retrieved pathogen with 46.8% of the total strains. In addition, *T. maritimum, E. cloacae*, and *S. iniae* pathogens were also isolated with 21.3%, 12.7%, and 19.2% of the total strains, respectively. The incidence of bacterial isolation from diseased *S. rivulatus* is demonstrated in Table "1".

On the other hand, a total of 46 pathogenic isolates were retrieved from moribund *R. haffara*. *Enterococcus faecalis* was the commonly recovered bacterial pathogen with 46.8% of the total isolates. In addition, *T. maritimum, E. cloacae*, and *S. iniae* pathogens

were also detected with 19.6%, 13%, and 19.6% of the total isolates, respectively. The incidence of bacterial isolation from diseased *R. haffara* is demonstrated in Table "2".

Table 1: Incidence of pathogenic bacteria retrieved from diseased *Siganus rivulatus*

Pathogenic bacteria	Spring		Summer		Winter		total isolates	
	No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%
T. maritimum	5	10.6%	3	6.4%	2	4.3%	10	21.3%
E. cloacae	2	4.3%	3	6.4%	1	2%	6	12.7%
E. faecalis	7	14.9%	12	25.5%	3	6.4%	22	46.8%
S. iniae	3	6.4%	4	8.5%	2	4.3%	9	19.2%
Total	17	36.2%	22	46.8%	8	17%	47	100%

Table 2: Incidence of pathogenic bacteria retrieved from diseased *R. haffara*.

Pathogenic bacteria	Spring		Summer		Winter		Total isolates	
	No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%
T. maritimum	5	10.9%	1	2.1%	3	6.5%	9	19.6%
E. cloacae	2	4.3 %	4	8.7%	0	0%	6	13%
E. faecalis	11	23.9%	7	15.2%	4	8.7%	22	47.8%
S. iniae	4	8.7%	3	6.5%	2	4.3%	9	19.6%
Total	22	47.8%	15	32.6%	9	19.6%	46	100%

Antibiogram

The tested *Enterococcus fecalis* isolates were sensitive to florfenicoL-FFC30 and mild sensitivity to erythromycin(E)-15, trimeth/sulfa-S XT25, and kanamycin-K30, while the same isolates showed resistant to ampicillin (10 µg), polymyxin-PB300 and tetracycline-TE30.

The results of antibiogram for the retrieved *Streptococcus iniae* isolates has revealed that isolates were only sensitive to erthromycin, florfenicol sulfamethoxazol-trimethoprim and mild sensitive to tetracycline, while resistant to polymyxin, ampicillin, and kanamycin. The results of antibiogram tests of *Enterococcus fecalis* and *Streptococcus iniae* are illustrated in Table "3".

Table 3: Antibiotic sensitivity patterns of *E. fecalis* and *S. iniae* isolates

Antibiotic	Standard inhibition zone (mm)		*Enterococcus fecalis*		*Strept .iniae*	
	Resistant	Sensitive	inhibition zone	Response	inhibition zone	Response
Erythromycin(E)-15.	< 14	>17	15 mm	Mild sensitive	28 mm	Sensitive
Trimeth/sulfa-S XT25	< 11	>15	12 mm	Mild sensitive	24 mm	Sensitive
Ampicillin-AMP10	< 12	>13	10 mm	Resistance	8 mm	Resistance
Florfenicol-FFC30	< 16	>21	25 mm	Sensitive	27 mm	Sensitive
Polymyxin-PB300	< 8	>12	5 mm	Resistance	7 mm	Resistance
Kanamycin-K30	< 14	>17	14 mm	Mild sensitive	9 mm	Resistance
Tetracycline-TE30	< 15	>18	8 mm	Resistance	17 mm	Mild sensitive

All tested *Enterobacter cloacae* strains were sensitive to ofloxacin(OFX)-5 and chloramphenicol (30), while these strains were resistant to erythromycin(E)-15., cephalothin(KF)-30, ampicillin-AMP10, and tetracycline-TE30. All the tested *Tenacibaculum maritimum* strains were sensitive to erythromycin(E)-15, cephalothin (KF)-30, ampicillin-AMP10, and chloramphenicol (30), while these isolates were resistant to ofloxacin(OFX)-5 and tetracycline-TE30. The results of antibiogram tests of *Enterobacter cloacae* and *Tenacibaculum maritimum* strains are shown in Table "4".

Table 4: Antibiotic sensitivity patterns of *E. cloacae* and *T. maritimum* isolates.

Antibiotic	Standard inhibition zone (mm)		*Enterobacter cloacae*		*Tenacibaculum maritimum*	
	Resistant	Sensitive	inhibition zone	Response	inhibition zone	Response
Erythromycin(E)-15	< 13	>23	18 mm	Resistance	30 mm	Sensitive
Cephalothin(KF)-30	< 18	>14	2 mm	Resistance	35 mm	Sensitive
Ampicillin-AMP10	< 13	>17	3 mm	Resistance	31 mm	Sensitive
Ofloxacin(OFX)-5	< 12	>16	20 mm	Sensitive	10 mm	Resistance
Chloramphenicol(30)	< 12	>18	25 mm	Sensitive	27 mm	Sensitive
Tetracycline-TE30	< 11	>15	10 mm	Resistance	9 mm	Resistance

DISCUSSION

Streptococcus spp. and *Enterococcus faecalis* are important fish bacterial pathogens that are incriminated in severe episodes accompanied by massive economic losses and substantial-high mortality in marine fish worldwide **(Austin and Austin., 2016).** Detection of fecal enterococci and other Enterobacteriaceae in fish samples collected from the Red Sea may be linked to sewage pollution **(Mahmoud *et al*., 2017).** Sewage and wastewater are the major sources of infectious agents. Contaminated water might encompass several bacterial pathogens, including flavobacterium, streptococcus, enterobacteria, and enterococci, with consequent health hazards to humans and fish **(Mahmoud *et al*., 2017).** In addition, sewage and municipal pollution are usually reducing fish immunity, rendering them more vulnerable to bacterial diseases.

In this study, a total of 250 Red Sea fishes were randomly caught from diverse regions along the coast of Hurghada city. The collected fishes were examined clinically for detecting bacterial pathogens. Moreover, different traditional techniques were used for the phenotypic characterization of the retrieved isolates. In addition, sequencing of the 16S rRNA locus was employed for genotypic characterization of the bacterial isolates. Additionally, the phylogenetic tree was constructed to identify bacterial strains recovered from marine fishes. Both fish species displayed clinical signs similar to septicemic bacterial diseases such as external hemorrhages, dark skin, scales detachment, fin rot,

skin erosions, ulcers, and ascites. The postmortem examination denoted several septicemic features signified by congestion and hemorrhages in different internal organs and enlargement of the liver and spleen. These results are following those formerly documented in several studies (**Mahmoud *et al.*, 2017; Elsayed *et al.*, 2018; Eissa *et al.*, 2021**). External and internal hemorrhages are the main clinical sign observed in stressed and diseased fish by septicaemic bacteria (**Fabbro *et al.*, 2011)**. Fishes under stressful marine ecosystems are more susceptible to bacterial outbreaks **(Hansen and Olafsen, 1999).**

Enterococcus faecalis and *S. iniae* have been documented to cause septicemic syndrome in wild marine fish with a high mortality rate. Several marine fishes have been highly susceptible to *E. fecalis* infections with high mortalities (**Rahman *et al.*, 2017**). Lethargy, exophthalmia, and hemorrhage on the skin and at the base of the fins are some of the clinical signs of infections (**Rahman *et al.*, 2017**). *E. faecalis* has scores of virulence factors, including lipoteichoic acid, which plays a significant role in the pathogenesis and inflammatory responses (**Park, 2013).**

API 20 E system is usually used to investigate the biochemical reactions of bacterial isolates. However, several molecular techniques have been developed for accurate and fast identification of pathogenic bacteria in farmed and wild fishes **(Abdelsalam *et al.*, 2017).** DNA-sequence-based identification mainly based on 16S rRNA and housekeeping genes **(Chatterjee and Haldar, 2012).** Sequencing of the 16S rRNA genes was demonstrated its usefulness in confirming the identification of the previous mentioned pathogenic bacteria, however this technique required expensive equipment render it less favorable in field diagnosis of fish diseases. These findings are coincided with the results obtained by **Eissa *et al.* (2015, 2020, 2021)** and **Essam *et al.* (2016),** who used 16S rRNA gene to identify *Vibrio* and photobacterium strains from moribund fishes.

Nucleotide phylogenetic analysis exhibited that both *E. faecalis* and *S. iniae* were clustered together in diverse branches with strong nodal of bootstrap. Interestingly, *E. cloacae* formed a monophyletic branch and separated from the streptococcus group.

CONCLUSION

Streptococcus, Enterococcus, Enterobacter, and *Flavobacterium* are pathogenic bacteria that isolated from the Red Sea fishes along with the Hurghada city. Streptococcosis and enterococcosis are pathogenic for humans and fish. Infectious agents such as *E. faecalis, S. iniae, E. cloacae,* and *T. maritimum* are highly risky pathogens that could cause septicemia and substantial mortality in wild fish. Polluted marine water was a likely source of these pathogenic bacteria.

REFERENCES

Abdelhak, E.; El Ganainy, A.; Madkour, F.; Abu El-Regal, M. and Ahmed, M. (2020). Comparative study on morphometric relationships and condition factor of *Siganus rivulatus* inhabits the Red Sea, Suez Canal and the Mediterranean Sea, Egypt. Egyptian Journal of Aquatic Biology and Fisheries, 24 (7):955-972.

Abdelsalam, M.; Abdel-Gaber, R.; Mahmoud, M.A.; Mahdy, O.A.; Khafaga, N.I. and Warda, M., (2016). Morphological, molecular and pathological appraisal of Callitetrarhynchus gracilis plerocerci (Lacistorhynchidae) infecting Atlantic little tunny (Euthynnus alletteratus) in Southeastern Mediterranean. Journal of advanced research, 7(2):317-326.

Abdelsalam, M.; Attia, M.M. and Mahmoud, M.A. (2020). Comparative morphomolecular identification and pathological changes associated with Anisakis simplex larvae (Nematoda: Anisakidae) infecting native and imported chub mackerel (Scomber japonicus) in Egypt. Regional Studies in Marine Science, 39:101469.

Abdelsalam, M.; Elgendy, M.Y.; Shaalan, M.; Moustafa, M. and Fujino, M., (2017). Rapid identification of pathogenic streptococci isolated from moribund red tilapia (*Oreochromis* spp.), Acta Veterinaria Hungarica. doi: 10.1556/004.2017.005

Abdelsalam, M.; Ewiss, M. Z.; Khalefa, H. S.; Mahmoud, M. A.; Elgendy, M. Y.; and Abdel-Moneam, D. A. (2021). Coinfections of *Aeromonas* spp., *Enterococcus faecalis*, and *Vibrio alginolyticus* isolated from farmed Nile tilapia and African catfish in Egypt, with an emphasis on poor water quality. Microbial Pathogenesis, 160: 105213.

Al-Niaeem, K. S.; Resen, A. K.; and Al-Haider, S. M. (2021). Potential of herbal extracts to avoid the bacterial infection of Enterobacter cloacae in common carp, Cyprinus carpio L. In IOP Conference Series: Earth and Environmental Science 779 (1): 012118.

Austin, B. and Austin, D.A. (2016). Bacterial fish pathogens. Diseases of farmed and wild fish, 6th Ed., Springer International Publishing, Switzerland.

Bridel, S.; Bourgeon, F.; Marie, A.; Saulnier, D.; Pasek, S.; Nicolas, P.; Bernardet, J.F.; and Duchaud, E. (2020). Genetic diversity and population structure of *Tenacibaculum maritimum*, a serious bacterial pathogen of marine fish: from genome comparisons to high throughput MALDI-TOF typing. Vet Res. 51(1):60.

Chatterjee, S. and Haldar, S. (2012). Vibrio Related Diseases in Aquaculture and Development of Rapid and Accurate Identification Methods. Marine Science Research and Development, 1:1-7.

Clinical and Laboratory Standards Institute (CLSI) (2014). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. CLSI Document M100-S24, Wayne, 34(1).

Di Cesare, A.; Vignaroli, C.; Luna, G.M.; Pasquaroli, S.; and Biavasco, F. (2012). Antibiotic-resistant enterococci in seawater and sediments from a coastal fish farm. Microb Drug Resist. 18(5):502-9.

Drancourt, M.; Bollet, C.; Carlioz, A.; Martelin, R.; Gayral, J. P. and Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. Journal of Clinical Microbiology, 38: 3623–30.

Eissa, A. E. (2016). Clinical and Laboratory Manual of Fish Diseases. LAP LAMBERT Academic Publishing.

Eissa, A. E.; Abdelsalam, M.; Abumhara, A.; Kammon, A.; Gammoudi, F. T.; Ben Naser, K. M.; Borhan, T. and Asheg, A. (2015). First record of *Vibrio vulnificus/Anisakis pegreffi* concurrent infection in black scorpionfish (*Scorpaena porcus*) from the South Mediterranean Basin. Research Journal of Pharmaceutical Biological and Chemical Sciences, 6(3): 1537-1548.

Eissa, A. E.; Abdelsalam, M.; Mahmoud, M. A.; Younis, N. A.; Mhara, A. A. and El Zlitne, R. A. (2020). Cutaneous fibropapilloma in Egyptian-farmed gilthead seabream (Sparus aurata; Linnaeus, 1758). Aquaculture International, 28(5): 2081-2091.

Eissa, A. E.; Abou-Okada, M.; Alkurdi, A. R. M.; El Zlitne, R. A.; Prince, A.; Abdelsalam, M. and Derwa, H. I. (2021). Catastrophic mass mortalities caused by *Photobacterium damselae* affecting farmed marine fish from Deeba Triangle, Egypt. Aquaculture Research. 52(9): 4455-4466.

EI-Jakee, J.; Elshamy, S.; Hassan, A.W.; Abdelsalam, M.; Younis, N.; El-Hady, M.A.; and Eissa, A. E. (2020). Isolation and characterization of mycoplasmas from some moribund Egyptian fishes. Aquaculture International, 28(3): 901– 912.

Elsayed, M.E.; Essawy, A.M.; Shabana, I.I.; Abou El-Atta, M.E. and EL-Banna, N.I. (2018). Studies on Bacterial Pathogens in Some Marine Fishes in ELMansoura, Egypt. American Journal of Agricultural and Biological Sciences, 13 (1): 9-15.

El-Sheshtawy, H.; Khalil, N.; Ahmed, W. and Abdallah, R. (2014). Monitoring of oil pollution at Gemsa Bay and bioremediation capacity of bacterial isolates with biosurfactants and nanoparticles. Marine pollution bulletin, 87: 191-200

Essam, H. M.; Abdellrazeq, G. S.; Tayel, S. I.; Torky, H. A. and Fadel, A. H. (2016). Pathogenesis of *Photobacterium damselae subspecies* infections in sea bass and sea bream. Microbial pathogenesis, 99: 41-50.

Fabbro, C.; Celussi, M.; Russell, H. and Del Negro, P. (2011). Phenotypic and genetic diversity of coexisting *Listonella anguillarum*, *Vibrio harveyi* and *Vibrio chagassi* recovered from skin haemorrhages of diseased sand smelt, *Atherina boyeri*, in the Gulf of Trieste (NE Adriatic Sea). Letters in Applied Microbiology, 54: 153–159.

Gabr, M.H.; Bakaili, A.S. and Mal, A.O., (2018). Growth, mortality and yield per recruit of the rabbit fish Siganus rivulatus (Forsskål 1775) in the red sea coast of Jeddah, Saudi Arabia. International Journal of Fisheries and Aquatic Studies, 6(1):87-96.

GAFRD. (2018). The General Authority for Fish Resources Development: Yearbook of fishery statistics (2018), Cairo, Egypt.

Habib, C.; Houel, A.; Lunazzi, A.; Bernardet, J.F.; Olsen, A.B.,7; Nilsen, H.; Toranzo, A.E.; Castro, N.; Nicolas, P. and Duchaud, E. (2014). Multilocus Sequence analysis of the marine bacterial genus *Tenacibaculum* suggests parallel evolution of fish pathogenicity and endemic colonization of aquaculture systems. Appl Environ Microbiol., 80(17):5503-14.

Hall, T.A. (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41: 95-98.

Hanna, R.G. (1995). An approach to evaluate the application of the vulnerability index for oil spills in tropical Red Sea environments. Spill Science & Technology Bulletin 2(2): 171-186.

Hansen, G.H. and Olafsen, J.A. (1999). Bacterial interactions in early life stages of marine cold water fish. Microbial Ecol. 38:1-26.

Kumar, K.; Prasad, P.; Raman, R.P.; Kumar, S. and Purushothaman, C.S. (2013). Association of Enterobacter cloacae in the mortality of *Pangasianodon hypophthalmus* (Sauvage, 1878) reared in culture pond in Bhimavaram, Andhra Pradesh, India. Indian J. Fish, 60(3): 147-149.

Kumar, S.; Stecher, G.; Li, M.; Knyaz, C. and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution, 35(6): 1547.

Lin, Y.J.; Rabaoui, L.; Maneja, R.H.; Pulikkoden, A.R.K.; Premlal, P.; Nazeer, Z.; Qurban, M.A.; Abdulkader, K.; Prihartato, P.K.; Qasem, A.M. and Fita, N., (2021). Strengths and weaknesses in the long-term sustainability of two sympatric seabreams (*Argyrops spinifer and Rhabdosargus haffara*, Sparidae). Journal of Fish Biology, 98(5):1329-1341.

Maiyza, S.; F Mehanna, S. and El-karyoney, I. (2020). An evaluation for the exploitation level of Egyptian Marine Fisheries. Egyptian Journal of Aquatic Biology and Fisheries, 24 (7): 441-452.

Mahmoud, M.M.; Ebtsam, S.H.; Essam, A.; Mohie, H.; Fatma, A.S. and Mahmoud, A. (2017). Bacterial infections in some red sea fishes, Assiut Vet. Med. J., 63(155): 86-93.

Mehanna, S. F.; El-Azim, H.A. and Belal, A. A. (2016). Impact of metal pollution, food availability, and excessive fishing on *Rhabdosargus haffara* stock (family: Sparidae) in Timsah lake. Environ. Sci. Pollut. Res., 23: 15888-15898.

Mohamed, M.A.E.; Madkour, H.A. and El-Saman, M.I. (2011). Impact of anthropogenic activities and natural inputs on oceanographic characteristics of water and geochemistry of surface sediments in different sites along the Egyptian Red Sea Coast. African journal of environmental science and technology, 5(7): 494-511.

Mustafa, G.A.; Abd-Elgawad, A.; Abdel Haleem, A.M. and Siam, R. (2014). Egypt's Red Sea coast: phylogenetic analysis of cultured microbial consortia in industrialized sites. Frontiers in microbiology, 5: 363.

Mustafa, G. A.; Abd-Elgawad, A.; Ouf, A. and Siam, R. (2016). The Egyptian Red Sea coastal microbiome: a study revealing differential microbial responses to diverse anthropogenic pollutants. Environmental pollution, 214: 892-902.

Osman, Y.; Mehanna, S.; El-Mahdy, S.; Mohammad, A. and Mahe, K. (2020). Age precision and growth rate of *Rhabdosargus haffara* (Forsskål, 1775) from Hurghada fishing area, Red Sea, Egypt. Egyptian Journal of Aquatic Biology and Fisheries, 24(2): 342-352.

Park, O.J., Han, J.Y., Baik, J.E., Jeon, J.H., Kang, S.S., Yun, C.H., Oh, J.W., Seo, H.S. and Han, S.H., (2013). Lipoteichoic acid of Enterococcus faecalis induces the expression of chemokines via TLR2 and PAFR signaling pathways. Journal of leukocyte biology, 94(6):1275-1284.

Rahman, M. S.; Kwon, W. S. and Pang, M. G. (2017). Prediction of male fertility using capacitation-associated proteins in spermatozoa. Molecular reproduction and development, 84(9): 749-759.

Rasul, N. M. A.; Stewart, I. C. F. and Nawab, Z. A. (2015). Introduction to the Red Sea: its origin, structure, and environment," in The Red Sea. Springer Earth System Sciences, eds N. Rasul and I. Stewart (Berlin: Springer), pp. 1–28. doi: 10.1007/978-3-662-45201-1_1.

Saleh, H.; Gabr, N.; Aboyadak, I. and Saber, N. (2019). Subcellular degenerative changes in hepatopancreas and posterior kidney of Streptococcus iniae infected Nile tilapia using Transmission Electron Microscope. Egyptian Journal of Aquatic Biology and Fisheries, 23(1): 305-316.

Sun, Y.; Wang, Z.; Fu, P.; Jiang, Q.; Yang, T.; Li, J. and Ge, X. (2013). The impact of relative humidity on aerosol composition and evolution processes during wintertime in Beijing, China. Atmospheric Environment, 77: 927-934.

Thillai Sekar, V.; Santiago, T. C.; Vijayan, K. K.; Alavandi, S. V.; Stalin Raj, V.; Rajan, J.J.S. and Kalaimani, N. (2008). Involvement of Enterobacter cloacae in the mortality of the fish, Mugil cephalus. Letters in applied microbiology, 46(6): 667-672.

Weisburg, W.G.; Barns, S.M.; Pelletier, D.A. and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of bacteriology, 173(2): 697-703.