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Molecular detection of the most common bacterial pathogens affecting economically important Egyptian Red Sea fishes

Asmaa W. Soliman[1], Mohamed Abdelsalam[2], Abbass Sayed Aboulezz[1]; Mahmoud Abou-Okada[2], Elsayed A. Eldessouki[3], Ghada O. El-Demerdash[4], Alaa Eldin Eissa[2]*

[1]National Institute of Oceanography & Fisheries (NIOF), Cairo, Egypt.
[2]Department of Aquatic Animal Medicine and Management, Faculty of Veterinary Medicine, Cairo University, Giza 12211 Egypt
[3]Agriculture Research Centre, Animal Health Research Institute, Port Said Provincial Laboratory, Egypt.
[4]Agriculture Research Centre, Animal Health Research Institute, Al-Fayoum Provincial Laboratory, Egypt.

*Corresponding Author: aeissa2012@cu.edu.eg

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ABSTRACT

The current study aimed to investigate the most common pathogenic bacteria that are naturally infecting wild marine fishes collected at different localities along the coastaline zone of Hurghada City, Egypt. A total of 300 samples of marbled spinefoot *Siganus rivulatus* and the Haffara Seabream *Rhabdosargus haffara* were subjected to clinical and bacteriological examinations. The examined fishes showed the characteristic clinical signs and postmortem lesions of vibriosis and photobacteriosis. Based on the morpho-chemical characterization, bacterial isolates retrieved from the naturally infected fishes were identified as *Vibrio* spp. and *Photobacterium* spp. Through sequencing 16S rRNA genes, the identities of bacterial isolates were confirmed as *V. alginolyticus*, *V. vulnificus*, *P. damselae* subsp. *damselae* and *P. damselae* subsp *piscicida*. *Vibrio alginolyticus* was the most frequent isolated bacterial pathogen and represented 54.4% and 46.7% of the total isolates recovered from *S. rivulatus* and *R. haffara*, respectively. Thus, the current study confirmed that *Vibrio* and *Photobacterium* species remain the most prevalent bacterial pathogens infecting Egyptian Red Sea fishes. From food safety perspective, these types of infections could pose potential public health hazards.

INTRODUCTION

The Red Sea is one of the largest semi-confined marginal maritime ecosystems worldwide that connects the Indian Ocean to the Mediterranean Sea **(Rasul *et al.*, 2015; Abdelsalam *et al.*, 2016).** The Red Sea has a unique biological, physical, geographical, and chemical characteristics. Egyptian coastline along the Red Sea is about 1080 Km **(Maiyza *et al.*, 2020)**. Although the Red Sea is considered one of the biggest marine

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biodiversity hotspots in the world, its aquatic ecosystems are still not fully understood. The Egyptian Red Sea encompasses economic important commercial fish species varied from pelagic, semi-pelagic, demersal, and benthopelagic **(Abdel-Azeem *et al.*, 2016)**. The Red Sea fisheries has a great socio-economic importance in terms of securing food resources, and as a source of income for tourism and fishing activities for Egyptians. However, the Red Sea has been unfortunately overexploited for decades in Egypt. The Red Sea coasts has been severely exposed to various pollution activities ascribed to industrialization, extensive fishing, tourism, human activities, maritime pollution and crude oil processing **(Mohamed *et al*., 2011)**. The Red Sea is one of the most heavily oil traffic waterways worldwide. Therefore, these serious environmental issues and the significant damage have great negative impacts on the Red Sea aquatic environment **(El-Sheshtawy *et al.*, 2014)**. The deterioration of the chemical, biological and physical characteristics of the Red Sea beyond acceptable limits could act as stress inducer and consequently compromises the immune barriers with consequent disease outbreaks.

Data extracted from two studies performed by **Eissa *et al*. (2021)** and **Mustafa *et al.* (2014)** suggested that coastal fish negatively respond to the impact caused by the anthropogenic pollution activities within aquatic ecosystems of the Red Sea. Such negative response may include pathogenic invasions and/or chemical pollution (heavy metals and crude oil pollution) **(Moustafa *et al.*, 2010*;* El-Moselhy *et al.*, 2014; Mustafa *et al.*, 2014, 2016)**. Previous studies dealt with the impact of anthropogenic pollution on the Egyptian Red Sea coasts with special reference to Hurghada concluded that several *Vibrio* species and *Photobacterium* species are prevailing in sediments and several marine species including fish and shellfish populations **(Abdel-Azeem *et al*., 2016; Mahmoud *et al.*, 2017**). Being pathogens of public health concern, the *Vibrio* and *Photobacterium* species have gained global interest of the microbiology community and public health experts **(Austin & Austin, 2016).**

Vibriosis is a worldwide aquatic animal disease that presents an actual danger for both aquatic species and human consumer. Several vibrios have been recorded to cause disease among marine fishes. The most frequently occurring vibrio infections in environmental samples and moribund marine fishes are *V. vulnificus, V. alginolyticus, V. parahaemolyticus,* and *V. anguillarum* **(Austin & Austin, 2016; Eissa *et al.*, 2017).** However, *V. parahaemolyticus, V. vulnificus,* and *V. alginolyticus* are more linked to fish and shellfish outbreaks **(Austin & Austin, 2016).** Vibrios are abundant in sediment substrates, marine environments and aquatic animals. They are free-living microbes and can survive the adverse conditions attributed to organic matter pollution **(Maugeri *et al.*, 2004)**. Interestingly, *Vibrio*s could easily attach and colonize the copepod integument in polluted and clean coastal zones.

Vibriosis induced septicemic clinical signs characterized by ascites, exophthalmia, external hemorrhages, anorexia and integumentary lesions in moribund fish **(Mahmoud *et al*., 2017)**. *Vibrio alginolyticus* and *V. vulnificus* are responsible for many outbreaks and mortalities among marine fishes which have high commercial value at the Red Sea communities **(Abdel-Azeem *et al*., 2016; Mahmoud *et al*., 2017**). Some *Vibrio*s have zoonotic potential due to the presence of virulence factors such as extracellular capsule polysaccharide (CPS), adhesive factor, hemagglutinins, hemolysin, protease, phospholipase, collagenase, enterotoxin and cytolysin **(Zhang & Austin, 2005; Hor & Chen, 2013)**. *Vibrio vulnificus* causes septicemia and cellulites among individuals handling moribund fishes, fishermen, and swimmers **(Al-Assafi *et al*., 2014; Austin & Austin, 2016)**.

On the other hand, *Photobacterium spp.* taxonomically belong to the genus *Photobacterium* that has been included in the family Vibrionaceae **(Eissa *et al.*, 2015; 2020)**. Across the Red Sea, *P. damselae* has been involved in mortalities among marine fishes **(Hashem, 2015)**. *Photobacterium damselae* subsp. *piscicida* is responsible for pasteurellosis in fish **(Mohamed *et al.*, 2016)**. Due to its growing numbers of outbreaks, this pathogen is considered a potential threat to fish abundance in the Red Sea basin **(Hashem, 2015)**. Further, *P. damselae* subsp *damselae* has a potential zoonotic significant causing human fatal infection **(Aigbivbalu & Maraqa, 2009)**.

The marbled spinefoot *Siganus rivulatus,* is a member of family Siganidae and is commonly known as Sigan, while the Haffara Seabream *Rhabdosargus haffara* is a member of family Sparidae that is commonly known as haffara. Both fish species are widely distributed across the Egyptian Red Sea with significant economic importance **(Abdelhak *et al.*, 2020; Osman *et al.*, 2020)**. Their high commercial values attracted great interests of consumers **(GAFRD, 2018; FAO, 2018)**. In Egypt, there has been a scarcity in literature discussing the *Vibrio* and *Photobacterium* infections among *S. rivulatus* and *R. haffara* populations throughout the entire coast of Hurghada city. Therefore, the current research aimed to determine and characterize the most common bacterial pathogens naturally infecting Red Sea fishes collected from different localities along the coastline zone of Hurghada city in Egypt. Besides, the phylogenetic tree was designed to clarify the identity of retrieved bacterial isolates.

MATERIALS AND METHODS

Fish specimens

A total number of three hundred fish specimens were collected by local fishermen along the coasts of Hurghada city of the Red Sea. Fish specimens were obtained regularly through the seasonal intervals between summer 2019 to spring 2020. The sampled fishes were marbled spinefoot *Siganus rivulatus* (Family: Siganidae, n = 150) and the Haffara Seabream *Rhabdosargus haffara* (Family: Sparidae, n = 150) (Table 1). Fishes were immediately transported to the National Institute of Oceanography and Fisheries (N.I.O.F.) in Hurghada, and in some cases transferred to the Lab of Aquatic Animal Medicine and Management, Cairo University using an insulated icebox. Fish specimens were identified and classified. Each fish specimen was assessed for the total length and weight. All national and institutional regulations for the use and care of fish were monitored.

Table 1. The number and scientific name of collected fish species

Fish species	Location	Number
marbled spinefoot *Siganus rivulatus*	Hurghada landing site	150 (50 in each season; summer, winter and spring)
Haffara Seabream *Rhabdosargus haffara*	Hurghada landing site	150 (50 in each season; summer, winter and spring)
Total		300

Clinical investigation

The collected fish were clinically investigated for any external lesions. In addition, fish samples were sacrificed and dissected for any gross lesions in the internal organs according to the method of **Eissa (2016)**.

Bacteriological examination

Loopfuls from brain, liver, kidney, and spleen were taken under aseptic conditions, and inoculated onto tryptic soya agar (TSA; Difco, Detroit, USA) , thiosulfate-citrate-bile salts-sucrose agar (TCBS; Difco, Detroit, USA), marine agar, blood agar and brain heart infusion agar. The inoculated plates were incubated at 25°C for up to 72 hrs. Portions of fish organs were inoculated into Alkaline Peptone Water (Difco) for enrichment and then streaked into both blood agar and TCBS agar and incubated at 25°C for 48-72 hrs. For isolates purification and identification, single colonies were selected and inoculated in BHI agar supplemented with 2% NaCl (v/v) (Difco). All used agar media were supplemented with 2% (w/v) NaCl (BHI; Difco, Detroit, USA). Suspected colonies were tested for Gram's stain, oxidase and catalase tests. Furthermore, motility on soft agar and sensitivity to vibrio static agent (O/129) were also examined. The identification of bacterial isolates was confirmed utilizing the API 20 E and API 20 NE Kit according to **Buller (2004)** and VITEK® 2 compact (BioMérieux), according to the guidelines of the manufacturer.

DNA extraction

The frozen bacterial isolates were revived from glycerol-preserved stocks and inoculated on BHI broth. Inoculated BHI broth tubes were incubated for 24 hrs at 25°C. Genomic DNA of purified isolates was extracted using PathoGene-spin™ DNA Extraction Kit according to the protocol of the extraction kit.

Sequencing of 16SrRNA gene

The amplifications of 16S rRNA gene were carried out through the following technique described by **Weisburg *et al*. (1991)**. The universal primer pairs (16S-F: -

AGA GTT TGA TCC TGG CTC AG) and (16S-R: - GGT TAC CTT GTT ACG ACT T) were used to amplify the 16S rRNA genes from different bacterial isolates. The PCR amplifications were performed in 50 µl total volume comprised of 25µl **M**axima® Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, MA، USA), 1ul 16S-F primer, 1ul 16S-R primer, 5 ul genomic DNA and 18 ul of nuclease-free water. The PCR assay was performed as the following steps: initial denaturation step at 95°C/ 7 min; followed by 35 cycles of 94°C/ 35 s, 55°C/ 35 s, and 72°C/ 45 s; and ended with final extension at 72°C/ 10 min. Amplicons were cleaned using GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA، USA) **(Abdelsalam *et al.*, 2009; 2015a, 2015b, 2017)**. Purified amplicons were electrophorized in 1.0% agarose stained with ethidium bromide, and finally, imagined under UV light. Purified amplicons were sequenced directly in both directions using ABI 3730xl DNA sequencer (Applied Biosystems™, USA) at Sigma Scientific Services Laboratory (Cairo, Egypt). The obtained sequences were checked and edited using Bio Edit (**Hall, 1999**).

Molecular identification

The assembled sequences of 16S rRNA genes of fish *Vibrio* and *Photobacterium* spp. isolates were submitted to the database of GenBank and consequently the accession numbers of these isolates was obtained and finally published in GenBank database. The BLASTN Search (National Center for Biotechnology Information, NCBI) was used to compare the assembled sequences against other related sequences deposited in GenBank. The criteria of 16S rRNA sequences-based assay for bacterial identification was explained by **Drancourt *et al*. (2000)**. Briefly, the bacterial isolates were ascribed to the genus and species level based on the similarity score values of 16S rRNA sequence. The bacterial identification to the species level was achieved when the similarity score value of 16S rRNA reached ≥99% to the relevant sequences in GenBank. While, the identification to the genus level was achieved when the similarity reached ≥97%. On the other hand, this method denoted that this bacterial isolate might belong to new genus and new species when the similarity score was below 97%.

Phylogenetic tree

The phylogenetic tree was constructed by Neighbor-Joining phylogeny test with 1000 bootstrap replicates using MEGA X (**Kumar *et al.*, 2018)**. The following factors were employed: substitutions: transversions and transitions, rate of variation among sites: uniform, and pattern among lineages: homogeneous. Different forms of convoluted tree are commonly available; however, this technique is adequate in positioning of current gram-negative bacteria at branch terminals. *Listeria monocytogenes* was selected as the out-group.

Antimicrobial sensitivity

The assessment of bacterial strains susceptibility to antibacterial agents was carried out by disc diffusion method **(Abu-Elala *et al.*, 2015)** on Muller–Hinton agar (Difco, Detroit, USA) using the following antimicrobial discs: ampicillin (10 ug), amikacin (30 ug), ciprofloxacin (5 ug), chloramphenicol (30 µg), cefotaxime (30 ug), doxycycline (30 µg), erythromycin (15 µg), florfenicol (30 µg), oxytetracycline (30 µg), polymixin (30 ug), streptomycin (10 ug), norfloxacin (10 ug) and sulfamethoxazole–trimethoprim (25 µg). The interpretation of inhibition zones was assessed in accordance with the specifications of the Clinical Laboratory Standard Institute Guidelines, **CLSI (2014)** and the bacterial strains described as susceptible, or resistant against the antimicrobial agents tested.

RESULTS

Clinical and post-mortem investigation

Clinical examination of the investigated *S. rivulatus* demonstrated skin darkening, boil-like furuncles scattered all over the body surface, ulcers in the head region, fin rot, and abdominal distension (Table 2 & Fig. 1), while the clinical signs of *R. haffara* displayed scattered external hemorrhages, scale detachments, erosions, and ulcers in the head region (Table 3 & Fig. 2). Remarkably, the post-mortem examination revealed moderate hemorrhage in the kidney, stomach and swim bladder. Moeover, moribund fishes revealed different septicemic clinical signs characterized by congestion and hemorrhages in gills and internal organs, splenomegaly, swollen of liver, and kidney and sometimes paleness of liver and intestine.

Table 2. Clinical and post-mortem examination of investigated *Siganus rivulatus*

Season	Number of fish	Percentage of fish	Clinical examination
Spring 2019	35	70%	Apparently normal fish
	15	30%	Fin erosions, multiple hemorrhages on skin with minimal ulcers, deep ulcer in the frontal area exposing underlying tissue, congested kidney, hemorrhagic and liquified brain
Summer 2019	30	60%	Apparently normal fish
	20	40%	Ulcerative dermatitis and dark skin, congested swim bladder, hemorrhage within brain tissues
Winter 2019	40	80%	Apparently normal fish
	10	20%	Fin rot

Table 3. Clinical and post-mortem examination of investigated *Rhabdosargus haffara*

Season	Number of fish	Percentage of fish	Clinical examination
Spring 2019	37	74 %	Apparently normal fish
	13	26 %	Congested fin and tail, thickens in swim bladder, and hemorrhage in swim bladder
Summer 2019	32	64 %	Apparently normal fish
	18	36 %	Congested liver, spleen and swim bladder, hemorrhagic kidney
Winter 2019	43	86 %	Apparently normal fish
	7	14 %	congested fins and gills

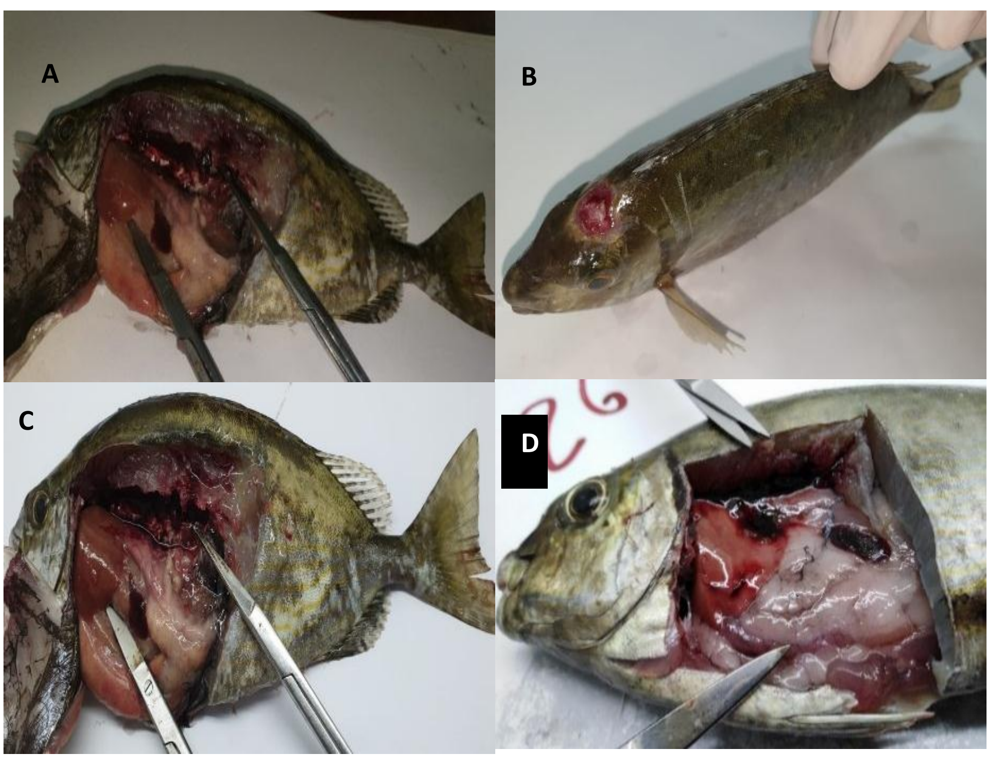

Fig. 1. A) Moribund *Siganus rivulatus* showing distended gall bladder. **B)** Ulcer and pore in the head region. **C)** Friable liver and severe congestion and hemorrhages in kidney. **D)** Severe congestion and hemorrhages in liver and kidney.

Fig. 2. A) Moribund fish showing ulcer in head region. **B)** Hemorrhages and erosion scattered in fish surfaces. **C)** Friable liver and severe congestion and hemorrhages in internal organs.

Bacterial examinations

Laboratory Microbiological Results

Four different bacterial pathogens were identified using ordinary and advanced molecular genetic techniques. The first identified bacterium was *Vibrio alginolyticus* isolates that appeared as 2-3 mm yellow colonies on TCBS. The isolated *V. alginolyticus* isolates were gram-negative motile, rods that were positive for catalase, oxidase, indole, H_2S, ornithine decarboxylase, and lysine. The Voges Proskauer reaction and methyl red test were positive. These isolates were negative for ß-galactosidase and arginine dihydrolase. These isolates degraded gelatin, chitin, lipids, blood, urea, and starch but not aesculin. They also reduced nitrates. They produced acid from mannitol, glycerol, maltose, salicin, sucrose, and mannose, but not from arabinose, lactose or inositol. These *V. alginolyticus* isolates yielded the following API E 20 profiles, (4 156 124), (4 242 124), (4 347 324) and (4 244 124).

The second identified bacterial pathogen was *V. vulnificus* that appeared 1-2 mm pin-point green colonies on TCBS. *Vibrio vulnificus* isolates were Gram-negative short rods, motile by a polar flagellum. They were positive for arginine dihydrolase, catalase and oxidase, Voges Proskauer reaction, but they were negative for ß-galactosidase, indole, lysine, ornithine decarboxylase. They degraded tween 80, casein lecithin, blood, and but not from urea and gelatin. Nitrates are reduced. They produced acid from cellobiose, D-amygdalin, D-galactose, D-fructose, D-glucose, maltose, melibiose, glycerol, mannose, trehalose and starch, but not from mannitol, arabinose, inositol, D-sorbitol, raffinose, inulin, sucrose and L-rhamnose. These *V. vulnificus* isolates yielded the following API E 20 profiles, (4 346 005), (5 306 005), (1 246 105) and (1 046 105). *Vibrio spp* are susceptible to vibriostatic agent (O/129) and Novobiocin (30 ug).

On the other hand, the third bacterial isolates were identified as *P. damselae subsp* *piscicida*, that appeared 1-2 mm, white and smooth colonies, pleomorphic rod-shaped, non-motile, bipolar staining and susceptible to vibriostatic agent (O/129). These isolates did not grow on TCBS and they were oxidase, Voges Proskauer reaction, and catalase positive, but they could not degrade urea. These *P. damselae subsp piscicida* isolates have an exceptional API E 20 profile, (2 005 004).

The fourth identified bacterial isolates were *P. damselae subsp damselae* that appeared as yellow and swarming colonies on TCBS. They were gram negative motile rod. They were positive for oxidase, catalase and Voges Proskauer reaction. They could degrade urea. These *P. damselae subsp damselae* isolates yielded the following API 20 E profiles, (2 012 004), (2 011 004), (2 010 004) and (2 010 024).

Molecular identification

The sequencing of 16s rRNA gene is regarded as golden device for the reconstruction of phylogenetic relationships and evolutionary history of pathogenic bacteria. The sequencing of 16S rRNA genes was conducted to confirm the identity of the retrieved bacterial isolates. The analysis of 16S rRNA genes sequences confirmed that two isolates belonging to *Vibrio* sp. were identified as *V. alginolyticus* and *V. vulnificus* and were submitted to GenBank database under the accession numbers MW508508 and MW508509, respectively. The GenBank accession no. (MW508508) was 1335-bp and showed 99.85% similarity to the accession number of *V. alginolyticus* (CP054700.1), and 99.78% similarity to the accession numbers of *V. alginolyticus* (MK308627.1, MZ045910.1, LC628646.1 and MH093761.1). While, the GenBank accession no. (MW508509) was 1295-bp and showed 99.69% similarity to the accession number of *V. vulnificus* (KT982478.1) and showed 99.61% similarity to the accession numbers of *V. vulnificus* (LC420074.1, MT052558.1, MK995604.1 and MG554519.1)

The analysis of 16SrRNA genes sequences confirmed that two isolates belonging to *Photobacterium* spp. were identified as *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* and were submitted to GenBank database under the accession numbers (MW508510 and MW508511), respectively. The GenBank accession no. (MW508510) was 1414-bp and showed 99.5-100% similarity to the accession numbers of *P. damselae* subsp. *piscicida* (MN186608, MT158694 and MW063536). While, the GenBank accession no. (MW508511) was 1487-bp and showed 99.80 - 100% similarity to the accession numbers of *P. damselae* subsp. *damselae* (MN120825, MN310924 and MN258947).

The phylogenetic analysis showed two major lineages. The first clade was further divided into two subclades with strong nodal support and 99% bootstrap value. The first subclade included *Vibrio* spp. isolates that clearly separated into *V. alginolyticus* isolates and *V. vulnificus* isolates forming a separate phylogenetic subclade with 99% bootstrap value. The current isolate of *V. alginolyticus* is embedded among other *V. alginolyticus* isolates and separated from *V. vulnificus*, and *V. parahaemolyticus* isolates. The second subclade included *P. damselae* subsp. *piscicida* isolates that grouped with other *P.*

damselae subsp. *damselae* isolates and was strongly supported by 100% bootstrap value and form a monophyletic group. The phylogenetic tree of the sequenced 16S rRNA genes of reterived bacterial isolates is demonstrated in Fig. 3.

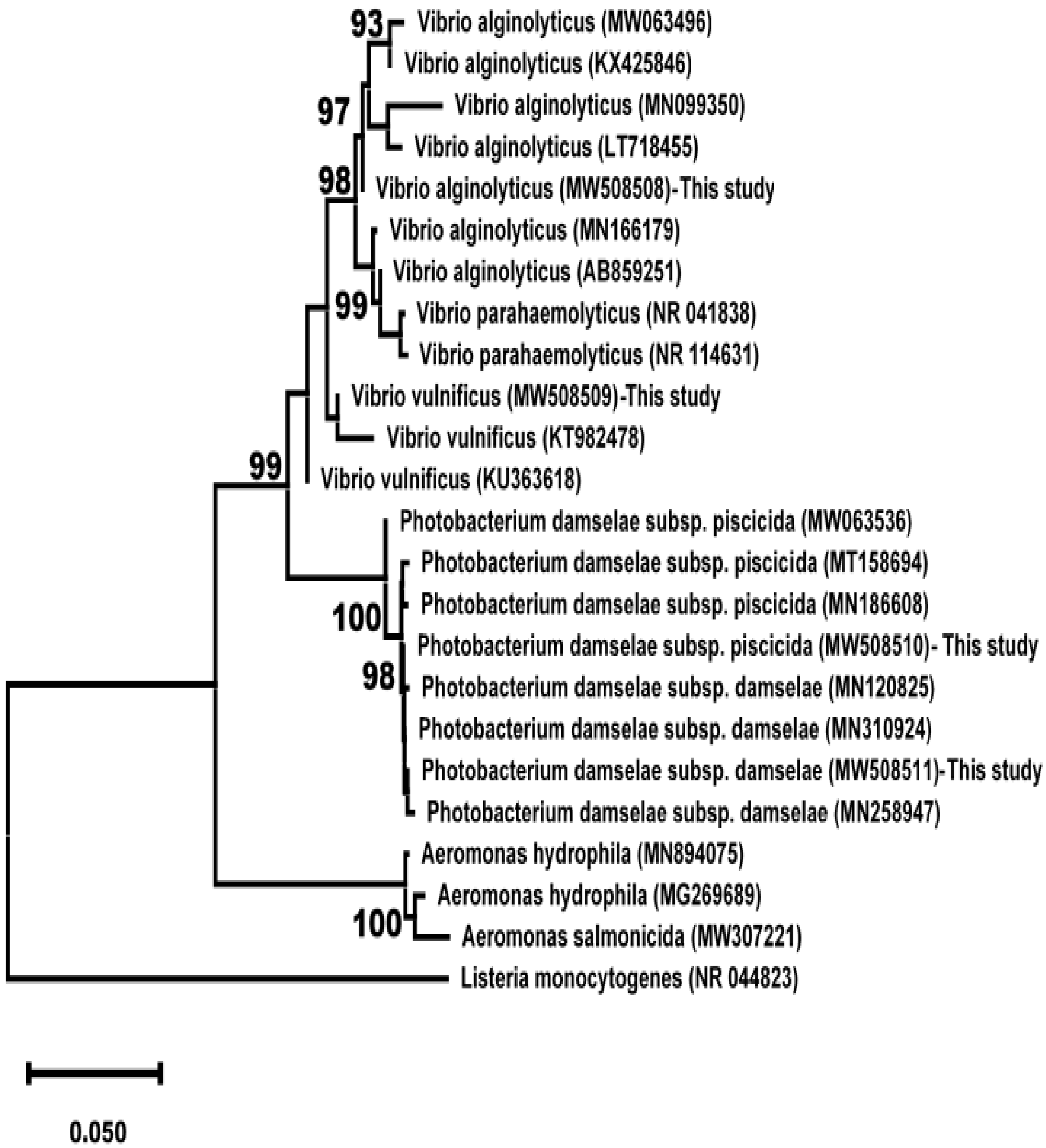

Fig. 3. The Phylogenetic tree based on neighbor-joining method demonstrated the comparative analysis of 16S rRNA gene sequences of *V. alginlyticus, V. vulnificus, P. damselae subsp. piscicida* and *P. damselae subsp. damselae* isolates with other closely related bacterial species.

Prevalence of bacterial isolates in *Siganus rivulatus*

A total of 46 bacterial strains were retrieved from moribund *Siganus rivulatus.* The most frequently isolated bacterial pathogens were *V. alginolyticus* with 54.4% of the total isolates. Other bacterial pathogens belonging to *V. vulnificus*, *P. damselae subsp damselae* and *P. damselae subsp piscicida* were also recovered with 21.7%, 17.4% and 6.5% of the total isolates, respectively. The frequency of bacterial isolates among moribund *Siganus rivulatus* is illustrated in Table (4).

Table 4. Percentages of retrieved isolates from moribund *Siganus rivulatus*

Bacterial isolates	Retrieved isolates in spring		Retrieved isolates in summer		retrieved isolates in winter		All isolates	
	Number	%	Number	%	Number	%	Number	%
V. alginolyticus	7	15.3 %	15	32.6%	3	6.5%	25	54.4%
V. vulnificus	3	6.5 %	5	10.9%	2	4.3%	10	21.7%
P. damselae subsp. *damselae*	1	2.2 %	7	15.2%	0	0%	8	17.4%
P. damselae subsp. *piscicida*	0	0%	3	6.5%	0	0%	3	6.5%
Total	11	24%	30	65.2%	5	10.8%	46	100%

Alternatively, a total of 45 bacterial strains were retrieved from diseased *Rhabdosargus haffara.* The *V. alginolyticus* were the most frequently isolated bacterial pathogens with 46.7% of the total isolates. Other bacterial pathogens belonging to *P. damselae subsp damselae, V. vulnificus*, and *P. damselae subsp piscicida* were also identified with 22.2%, 20% and 11.1% of the total isolates, respectively. The frequency of bacterial isolates among moribund *Rhabdosargus haffara* is illustrated in Table (5).

Table 5. Percentages of retrieved isolates from moribund *Rhabdosargus haffara*

Bacterial isolates	Retrieved isolates in spring		Retrieved isolates in summer		Retrieved isolates in winter		All isolates	
	Number	%	Number	%	Number	%	Number	%
V. alginolyticus	6	13.3 %	13	28.9%	2	4.5%	21	46.7%
V. vulnificus	3	6.7 %	6	13.3%	0	0%	9	20%
P. damselae subsp. *damselae*	2	4.4 %	7	15.6%	1	2.2%	10	22.2%
P. damselae subsp. *piscicida*	0	0%	4	8.9%	1	2.2%	5	11.1%
Total	11	24.4%	30	66.7%	4	8.9%	45	100%

Antibiogram

The tested *V. alginolyticus* isolates were sensitive to sulfamethoxazole-trimethoprim (25ug), doxycycline (30 ug) and polymyxin (30 ug), while the same isolates showed resistance to ampicillin (10 ug), oxytetracycline (30ug) and florfenicol (30 ug). In contrast, the tested *V. vulnificus* isolates showed antibiotic sensitivity to doxycycline (30ug) and polymyxin (30 ug), while these isolates were resistant to sulfamethoxazole-trimethoprim (25 ug), ampicillin (10 ug), oxytetracycline (30 ug) and florfenicol (30 ug). The results of antibiogram tests of *V. alginolyticus* and *V. vulnificus* are illustrated in Table (6).

All tested *P. damselae subsp. piscicida* strains were sensitive to amikacin (30ug), ampicilin (10ug), oxytetracycline (30ug), streptomycin (10ug), and erythromycin (15ug), while these strains were resistant to chloramphenicol (30ug), cefotaxime (30ug), norfloxacin (10ug), and ciprofloxacin (5ug). All the tested *P. damselae subsp. damselae* strains were sensitive to amikacin (30ug), oxytetracycline (30ug), erythromycin (15ug), chloramphenicol (30ug), norfloxacin (10ug), and ciprofloxacin (5ug), while these isolates were resistant to ampicilin (10ug), streptomycin (10ug), and cefotaxime (30ug). The results of antibiogram tests of *P. damselae subsp. piscicida* and *P. damselae subsp. damselae* strains are shown in Table (7).

Table 6. Antibiotic sensitivity patterns of *V. vulnificus* and *V. alginolyticus* isolates

Antibiotic	Standard inhibition zone		*Vibrio vulnificus*		*Vibrio alginolyticus*	
	Resistant	Sensitive	Inhibition zone	Response	Inhibition zone	Response
Sulfamethoxazole-trimethoprim -25ug	≤ 11	≥ 15	6 mm	Resistant	16 mm	Sensitive
Ampicilin-10ug	≤ 12	≥ 13	0 mm	Resistant	1 mm	Resistant
Oxytetracycline-30ug	≤ 15	≥ 18	15 mm	Resistant	11 mm	Resistant
Doxycyclin-30ug	≤ 8	≥ 12	16 mm	Sensitive	24 mm	Sensitive
Florfenicol-30ug	≤ 16	≥ 21	10 mm	Resistant	12 mm	Resistant
Polymixin-30ug	≤ 8	≥ 12	17 mm	Sensitive	19 mm	Sensitive

Table 7. Antibiotic sensitivity patterns of both *subsp* of *Photobacterium damselae* isolates

Antibiotic	Standard inhibition zone (mm)		*P. damselae subsp. damselae*		*P. damselae subsp. piscicida*	
	Resistant	Sensitive	Inhibition zone	Response	Inhibition zone	Response
Amikacin-30ug	≤14	≥ 17	22mm	Sensitive	19mm	Sensitive
Ampicilin-10ug	≤13	≥ 17	10mm	Resistant	18mm	Sensitive
Oxytetracycline-30ug	≤ 15	≥18	24mm	Sensitive	19mm	Sensitive
Streptomycin-10ug	≤11	≥ 15	9mm	Resistant	22mm	Sensitive
Chloramphenicol-30ug	≤12	≥ 18	23mm	Sensitive	0mm	Resistant
Cefotaxime-30ug	≤22	≥ 26	17mm	Resistant	15mm	Resistant
Erythromycin-15ug	≤13	≥ 23	25mm	Sensitive	24mm	Sensitive
Norfloxacin-10ug	≤12	≥ 17	26mm	Sensitive	9mm	Resistant
Ciprofloxacin-5ug	≤15	≥ 21	27mm	Sensitive	9mm	Resistant

DISCUSSION

Disease outbreaks caused by *Vibrio* and *Photobacterium* species are considered a common phenomenon that leads to significant mortality and huge economic losses in marine fish globally **(Buller, 2004; Storm *et al.*, 2013; Austin & Austin, 2016)**. Photobacterium and vibrios are widely distributed in the marine ecosystems and sediments, integumentary system and in the intestinal contents of aquatic marine vertebrates and invertebrates **(Thompson *et al.*, 2004; Eissa *et al*., 2021)**. Vibrios are gram-negative, motile, non-spore forming rods belonging to Vibrionaceae family within the class Gammaproteobacteria **(Chatterjee & Haldar, 2012; Storm *et al.*, 2013; Al-Assafi *et al*., 2014)**. While, photobacterium are gram-negative rods, motile by a polar flagellum, oxidase positive belonging to Vibrionaceae family **(Hashem, 2015; Mohamed *et al*., 2016; Mahmoud *et al.*, 2017; Eissa *et al.*, 2021).** These facultative anaerobic bacteria are very common in the aquatic environments and have wide range of susceptible host **(Eissa *et al.*, 2015, 2020, 2021)**. Regardless of the common contributions of different *Vibrio* or *Photobacterium* species that cause diseases in aquatic animals, *V. alginolyticus, V. vulnificus, P. damselae subsp. damselae and P. damselae subsp. piscicida* are the leading causes of disease outbreaks in marine fishes resulting in food insecurity and economic casualties globally **(Chatterjee & Haldar, 2012; Mustafa *et al.*, 2014, 2016; Elsayed *et al.*, 2018)**.

In the current study, a total of 300 marine fishes were recovered from several localities along the coastline of the Red Sea in Hurghada city. These marine fishes were subjected to clinical, postmortem examination and screening for the incidence of pathogenic bacteria. In addition to the morpho-biochemical characterization of the bacterial strains, the molecular identification was also performed by sequencing the 16S rRNA genes. Furthermore, the phylogenetic relationships were executed to confirm the identity of bacterial strains isolated from moribund fishes. Clinical examination of different fish species revealed skin darkness, external scattered hemorrhages, boil-like (furuncle) lesions, sloughing of fish scales, erosions, ulcers in the head region, fins rot, and abdominal distension. The collected marine fishes were further investigated for postmortem changes of the internal organs. The necropsied fishes revealed clear signs of septicemia manifested by gills congestion and viscera, enlargement of different internal organs, and some cases exhibited liver paleness and intestine. These findings agree with those previously reported in many studies (**Mustafa *et al.*, 2014, 2016; Hashem, 2015; Mohamed *et al*., 2016; Mahmoud *et al.*, 2017; Elsayed *et al.*, 2018; Eissa *et al.*, 2021**).

Hemorrhages are the predominant clinical sign noticed in stressed fishes and in septicemic bacterial infection in fish (**Fabbro *et al.*, 2011)**. This relationship indicates that septicaemic bacterial pathogens, such as *Vibrio* and *Photobacterium* species have the ability of causing infection only when the fish are exposed to stress factors **(Moustafa *et al.*, 2010)**. On the other hand, Hurghada coastline is experienced to adverse environmental pollution due to anthropogenic activities, including landfilling, shipping operation, touristic activities, sewage pollution, and drainage of desalination plants (**Madkour & Dar, 2007; Abdel-Azeem *et al.*, 2016)**. It is worth mentioning that, the aquatic animals under stressful environmental condition are more prone to bacterial infections **(Hansen & Olafsen, 1999)**. Vibriosis and photobacteriosis are usually linked to water contamination and stress leading to disease outbreaks **(Eissa *et al.*, 2015; Zhang & Austin, 2005)**. Although fish vibriosis and photobacteriosis could be presumptively diagnosed through the pathognomonic clinical lesions, the confirmatory diagnosis of the infection requires the isolation followed by morpho-chemical and molecular characterization of the causative agent **(Eissa *et al*., 2016)**.

Isolation and identification of *Vibrio* and *Photobacterium* strains depend on the colonial characters on selective media and the phenotypic, biochemical and enzymatic characterization. The different bacterial isolates retrieved from naturally infected fish were presumptively identified as *V. alginolyticus*, *V. vulnificus*, *P. damselae subsp picicida* and *P. damselae subsp damselae* depending on the phenotypic and biochemical characterization including the API 20 E profiles. These outcomes are in accordance with that described previously in many studies **(Buller, 2004; Chang *et al.*, 2011; Abdel-Aziz *et al.*, 2013; Austin & Austin, 2016**). Notably, the 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 **(Abdelslam *et al.*, 2017)**. The DNA-sequence-based identification is mainly based on 16S rRNA and housekeeping genes **(Chatterjee & Haldar, 2012)**. Sequencing of the 16S rRNA genes has proven its usefulness in confirming the identification of the previously mentioned pathogenic bacteria, however this technique requires expensive equipment which renders it less favorable in the 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 *V. alginolyticus* and *V. vulnificus* were clustered together in diverse branches with strong nodal of bootstrap. Interestingly, both subsp. of *P. damselae* were grouped together in the same branch and separated from *Vibrio* species with high bootstrap value. These results proved that the two clusters of *Vibrio* and *Photobacterium* isolates belonged to one family; Vibrionaceae.

A previous study of the Egyptian Red Sea sediments confirmed the isolation of some pathogenic bacteria including *V. alginolyticus* and *V. vulnificus* **(Mustafa *et al*., 2014)**. In this study, two different species of *Vibrio* spp. and two subspecies of *Photobacterium damselae* have been reported in moribund marine fishes and this coincides with the findings of **Moustafa *et al.* (2010)** who reported that, *V. alginolyticus* was associated with vibriosis in the Red Sea marine fish. In addition, 80.4% of shrimps from Suez are infected by *V. alginolytcus* **(Abd El-baky, 2012)**. Interestingly, *V. alginolytcus* was isolated from Bird wrasse fish; *Gomphosus varius;* collected from the indoor aquarium of National Institute of Oceanography (NIOF) in Hurghada **(El-Galil & Mohamed, 2012)**. These reports agree with the present study, which revealed that *V. alginolyticus* was the most frequent bacteria isolated from moribund Red Sea fishes.

CONCLUSION

Vibrios and *Photobacterium* are the most retrievable pathogens from the naturally infected and economically important fishes along the Hurghada coastline of the Red Sea. Vibrios are pathogenic for both human and aquatic animals. Mostly, these pathogens are considered as potential biological indicator of sewage and municipal pollution that is tightly linked to the touristic activities along the coast of Hurghada. This necessitates an urgent awareness of the governmental and NGO to adopt the most reliable hygienic measures to combat coastal pollution along the Red Sea.

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