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Monitoring of different *vibrio* species affecting marine fishes in Lake Qarun and Gulf of Suez: Phenotypic and molecular characterization

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ABSTRACT

Vibriosis is a globally threatening bacterial disease affecting mariculture with high mortalities and severe economic losses. Isolation and Identification of different *vibrio* species were performed to a total number of one hundred moribund and freshly dead *Solea aegyptiaca, Epinephelus marginatus* and *Mugil cephalus* collected from Lake Qarun and Gulf of Suez. Phenotypic picture and molecular identification based on use of 16SrRNA gene sequence confirmed 44 strains as *vibrio* species. Further molecular identification of retrieved *vibrio spp.* using species specific primers for collagenase, ToxR and Vvh genes categorized 10 isolates belong to *V. alginolyticus*, 8 isolates belong to *V. parahaemolyticus* and 6 isolates belong to *V. vulnificus*. The total prevalence of vibriosis was 44% where the highest prevalence was recorded in Lake Qarun examined fishes.

Introduction

In Egypt, Mariculture represents an important investment for fishermen, but diseases and high feeding cost are the main obstacles facing the sustainability and profitability of this sector (Khalil and Abd El-Latif, 2013). Vibriosis is one of the devastating bacterial diseases affecting marine fishes, crustaceans and bivalves causing high mortalities with severe economic losses worldwide (Sarjito et al. 2009; Samuelsson et al. 2006). Family *Vibrionaceae* contain 45 species of Gram-negative gamma proteobacteria that live in a wide range of aquatic environments (Ruwandeepika et al., 2012). They are found in marine, estuarine and fresh ecosystems, constituting about 60% of total heterotrophic bacteria. Moreover, they represent a part of the normal fish flora in the aquatic organisms that could act as vectors for the disease transmission (Sonia and Lipton, 2012; Crisafi, 2010). *Vibrio* is a halophilic, facultative aerobic, Gram-negative bacterium that is strongly correlated with high salinity (30–35 ppt), high temperature, parasitic infestation and mechanical injuries. These factors suppress the immunity and increase the susceptibility of fish to vibriosis (Nagasawa and Cruz-Lacierda, 2004; Haenen et al., 2014 and El-Bouhy et al., 2016). Different types of *Vibrio* (*V. anguillarum, V. alginolyticus, V. cholera, V. damsela, V. ordalii, V salmonicida, V. parahaemolyticum* and *V. vulnificus*) were isolated from different cultured and wild marine fish species (Akhlaghi, 1999; Magariños et al., 2011 and Tapia-Paniagua et al., 2012). Invasion and proliferation of *Vibrio* species in aquatic host depend mainly on chemotactic motility of the bacteria followed by deployment of Sidrophore-mediated iron-sequestering system to "steal" iron from the host and finally production of extracellular products as collagenase, cytotoxins, enterotoxins, lytic enzymes, protease, chondroitin sulfatase, amylase, and different hemolysins, which eventually caused typical hemorrhagic septicemia (Larsen and Boesen, 2001; Naka et al., 2013 and Toranzo, 2004). Literature concerning typing and epidemiological mapping of *vibrio* species affecting *Solea aegyptiaca, Epinephelus marginatus* and *Mugil cephalus* is scarce. So, this study was planned for monitoring of different *vibrio* species isolated from *Solea aegyptiaca, Epinephelus marginatus* and *Mugil cephalus* collected from Lake Qarun and Red sea with phenotypic and molecular characterization of isolated species. 16SrRNA was used as housekeeping gene for detection of *vibrio* species. Further molecular identification was adopted using species specific primers.

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Materials and methods

Fish samples

A total of one hundred (100) wild moribund and freshly dead *Solea aegyptiaca, Epinephelus marginatus* and *Mugil cephalus* were collected in the period from October 2014 to July 2015 from Shakshouk area, Lake Qarun at Fayoum province and Gulf of Suez, Egypt. The collected fish samples were submitted directly to the lab for clinical and bacteriological examination (Buller, 2004; Austin and Austin, 2012).

Clinical and postmortem examination

External and internal examination of fish samples were performed to record clinical abnormalities according to (Austin and Austin, 2012).

Bacteriological examination

Kidney and liver samples were taken under complete aseptic conditions, and striked directly on TCBS (thiosulphate citrate bile salt agar, OXOID) plates. All plates were incubated at 28 °C for 18–24 hr. Ordinary biochemical identification of bacterial isolates were performed using motility, Gram's stain, oxidase and catalase tests followed by Analytical Profile index 20NE (Biomerieux) (Buller, 2004). The purified strains were stored in BHI + 15% (vol/vol) glycerol at −20 °C.

Molecular identification and Partial sequences of 16SrRNA gene

The retrieved *vibrio* species were cultured on tryptic soya agar +2% NaCl for genomic DNA extraction using Prepman® Ultra Reagent (Applied Biosystems, Massachusetts) according to the manufacturer procedures. The reaction for PCR amplification of each sample was performed in a total volume of 25 ul containing: 12.5 µl PCR master mix (Qiagen), which provides 2.5 units per reaction of DNA polymerase, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1X PCR buffer (with 1.5 mM MgCl2), 8.5 µl PCR grade water, 1 µM of each primer (10 pmol) and 2 µl Template DNA. Internal fragment of the 16SrRNA gene was amplified using primer sets designed by (Montieri et al., 2010). Collagenase, toxR and Vvh genes were used for species specific detection of *V. alginolyticus, V. parahaemolyticus and V. vulnificus* respectively (Di Pinto et al., 2005 and Kim et al., 1999). tdh gene was used for confirming the pathogenicity of different *vibrio* isolates. The primer sets and the cycling conditions used in this study are described in Tables 1 and 2. The amplified fragment of 16SrRNA gene of *vibrio* species were sequenced using Genetic Analyzer 3500 (Applied Biosystems), and blasted at the NCBI BLAST home page (https://blast.ncbi.nlm.nih.gov/Blast/).

Water quality parameters

Water Salinity and temperature were measured using Waterproof digital combo [HI 98127 (pHep 4) -Hanna instruments Inc., RI, USA] containing Thermometer and Refractometer.

Experimental infection

A total number of 120 apparently healthy *Oreochromis niloticus* were acclimated in 24 glass aquaria for 2 weeks before the experimental infection. 0.2 ml of 5×10^7 cfu/ml, of *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus* bacterial suspension were intraperitoneally injected for detecting the potential pathogenic isolates (Li et al., 2016), mortalities and clinical abnormalities were recorded daily for one week post injection. Freshly dead fish were subjected to bacteriological examination for bacterial reisolation. The mortality percentages were calculated as following

$$MR\% = \frac{\text{number of death in specified period}}{\text{Total number of population}} \times 100 \quad (1)$$

Results

Clinical signs and Necropsy findings of naturally infected marine fishes

The common clinical signs recorded were dark coloration of skin with detached scales, Hemorrhages at the base of the fins

Table 1
The primer sets used in this study.

Vibrio primer	Oligonucleotide sequences (5′-3′)	Length of amplified product	Reference
16SrRNA	F: CAGGCCTAACACATGCAAGTC R:GCATCTGAGTGTCAGTATCTGTCC	700 bp	Montieri et al. (2010)
Collagenase gene	F:CGAGTACAGTCACTTGAAAGCC R: CACAACAGAACTCGCGTTACC	737 bp	Di Pinto et al. (2005)
Tdh gene	F: CCATCTGTCCCTTTTCCTGC R: CCAAATACATTTTACTTGG	373 bp	Mustapha et al. (2013)
ToxR gene	F: GTCTTCTGACGCAATCGTTG R: ATACGAGTGGTTGCTGTCATG	368 bp	Kim et al. (1999)
Vvh gene	F:CGCCGCTCACTGGGGCAGTGGCTG R:CCAGCCGTTAACCGAACCACCCGC	387 bp	Brauns et al. (1991)

Table 2
cycling conditions of used primers.

Gene	Initial denaturation	Amplification			
		Denaturation	Annealing	Extension	Final extension
16SrRNA (30cycle)	95 °C for 10 min	95 °C for 1 min	55 °C for 1 min	72 °C for 1.5 min	72 °C for 5 min
Collagenase Gene (35cycle)	95 °C for 15 min.	94 °C for 30sec.	57 °C for 30sec	72 °C for 1 min	72 °C for 5 min
Tdh gene (30cycle)	94 °C for 5 min.	94 °C for 1 min.	54 °C for 1 min	72 °C for 1 min	72 °C for 5 min
ToxR gene (20 cycle)	94 °C for 10 min.	94 °C for 1 min	63 °C for 1.5 min	72 °C for 1.5 min	72 °C for 10 min
Vvh gene (4o cycle)	94 °C for 10 min.	94 °C for 1 min	65 °C for 1 min	65 °C for 1 min	65 °C for 10 min

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