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Isolation of *Vibrio alginolyticus* and *Vibrio vulnificus* Strains from Cultured *Oreochromis niloticus* Around Qarun Lake, Egypt

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Abstract: *Vibrio alginolyticus* and *Vibrio vulnificus* were isolated from Nile tilapia fishes (*Oreochromis niloticus*) in a private farm nearby Qarun Lake without fish morbidity or mortality documented. The water parameters showed rise in ammonia and water salinity. Isolates were biochemically and molecularly identified using primers for 16srRNA. *V. alginolyticus* and *Vibrio vulnificus* were isolated from 87.5% and 12.5% of the examined fishes respectively. The *V. Alginolyticus* isolates were pathogenic to Nile tilapia with LD_{50} ($1x10^6$). The isolated strains were sensitive to cefotaxime, streptomycin and chloramphenicol.

Key words: *Oreochromis niloticus · Vibrio alginolyticus · Vibrio vulnificus*

INTRODUCTION

Tilapia represents the second largest group of fish produced from aquaculture after Cyprinidae in the world. Among tilapia species, the *Nile tilapia* (*Oreochromis niloticus*) is the most widely cultured because of its fast growth, ability to reproduce in freshwater and saline water and easily cultivation in ponds, rivers or dams [1].

The vibrios are Gram-negative rod-shape bacteria, catalase and oxidase positive, motile bypolar flagella and sensitive to the vibriostatic agent O/129. Vibrios are ubiquitous to marine environment and are widely distributed in the coastal seawaters and /or brackish waters. The clinical disease outbreaks only occur when fish exposed to infectious agent in existence of sharply stressed factors [2-4].

Vibrio vulnificus causes severe wound infections and life-threatening septicemia in human. Also, recovered from oysters and fish and classified into three biotypes based on growth characteristics and serology [5-7]. *V. alginolyticus* frequently isolated from outbreaks and mortalities in many marine fish species including Carpet Shell Clam Larvae, gilt-head sea bream, silver sea bream, Gilt-head sea bream, cultured black sea bream fry and sturgeon (*Acipenser baerii*) [3, 4]. In addition, *V. alginolyticus* causes many epizootic outbreaks among the Gilt-head seabream and European seabass populations in Mediterranean countries [8, 9] and from some Egyptian coastal provinces [10].

Therefore, this study was carried out with an objective to provide information on the presence of *Vibrionaceae* (*V. alginolyticus* and *Vibrio vulnificus*) in Nile tilapia from a private brackish water farm near Qarun Lake, Fayoum governorate, Egypt.

MATERIALS AND METHODS

Bacterial Sample: A total of 600 Nile tilapia fish (*Oreochromis niloticus*) were purchased from a private farm in Al Fayoum governorate, near Qarun Lake for experimental trials in our Centre. All fish randomly collected were apparently healthy as evidenced by the absence of any external abnormalities such as lesions and farm didn't record any outbreaks except normal sporadic mortality. The mean body weights of the fishes collected was 100±10 gm. All fishes were transferred to clean water tanks (100 L) in our lab. No mortality was observed at that time. By the next day, all fish were lethargic and death started to appear within all fish tanks. By the second day from purchase all fish except 30 died.

Moribund and recently died fish were collected and subjected to clinical and postmortem examinations.

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Bacterial Isolation and Phenotypic Characterization: Samples for bacterial isolation were taken from kidney of moribund and freshly died fish (n= 40) and cultured on Tryptic soy broth (TSB) (Lab M, UK), plates of tryptic soy agar medium (TSA) (Lab M, UK), *Aeromonas* agar media with ampicillin supplement and Thiosulphate citrate bile salt sucrose agar (TCBS, Lab M, UK). The media were prepared with 2% NaCl. The inoculated plates were incubated at 25°C for up to 48h.

The purified colonies were phenotypic characterized using commercial API20NE kit (BioMerieux, France) according to the manufacturer's instructions.

Water Samples: Water samples were taken from the farm in dark brown clean and dry bottles. Water temperature, pH, salinity and Dissolved Oxygen (DO) were measured. While, ammonia, nitrates and iron were determined in laboratory according to methods described previously in (APHA) [11].

DNA Extraction: The colonies were grown on TSA medium supplemented with 2% sodium chloride and incubated at 25°C for 24 h. Bacterial colonies were harvested. The extraction of genomic DNA was carried out according to Fermantas DNA extraction kit manufacturer's instructions.

Two sets of oligonucleotide primers were used for identification general 16s rRNA. The eubacterial universal pair of primers were 63f (5'-CAGGCCTAACACATGCAAGTC) and 1387r (5'-GGGCGGWGTGTACAAGGC) to generate an amplified product of 1.3 kb as previously described in Marchesi, *et al*., [12].

The amplifications products were purified using spin Columns (Fermantas, Lithuania) for direct sequencing. DNA sequencing was carried out by using Sanger DNA sequencer (Applied Biosystems, USA). NCBI nucleotide collection (nr/nt) databases search using BLAST tool was performed for purpose of verifying the amplified products.

Determination of LD_{50} dose: Nile tilapia fish were acclimated for one week with no history of infection under laboratory conditions. The strain was grown to mid-log phase and bacteria enumerated by plate dilution method. Different concentrations of the bacteria ($1x10^3$ to $1x10^{10}$) injected intra-peritoneal into each group of fish (n= 5) and observed for 14 days, the clinical signs and numbers of dead fish were recorded during the observation time and LD_{50} was calculated according to Reed and Muench, [13].

Antibacterial Susceptibility: The antimicrobial susceptibility of the *V. alginolyticus* isolates was determined by the disc diffusion method on Mueller Hinton Agar as described by the Clinical and Laboratory Standards Institutes [14]. The antibiotic discs used in this study were purchased from Sigma. The following antibiotics were tested: amoxicillin, oxytetracycline, trimethoprim–sulfamethoxazole, streptomycin, Kanamycin, erythromycin, novobiocin, chloramphenicol, nalidixic acid and cefotaxime.

RESULTS

Clinical Signs: The clinical signs of the diseased fish were lethargic, skin depigmentation and hemorrhagic spots. The main postmortem lesions were congestion of liver, spleen and stomach and ascetic fluid in the intestine. The mortality among the diseased fish was 95%.

Water Quality: The results of this study revealed the elevation of ammonia and pH values and decrease of dissolved oxygen in the water samples. The recorded water quality results were indicating rise in un-ionized ammonia (9.0 mg/l), water salinity (4.3 g/l), nitrite (5.9 mg/l), iron (2.1 mg/l) with concurrent decrease in dissolved oxygen (3.9 mg/l) (Table 1).

Isolation and Characterization of the Bacterial Strains: The results revealed that the total prevalence of bacterial *Vibrio* spp., isolates from apparently healthy *O. niloticus* in this study was 87.5%. Species wise, the total prevalence of *V. alginolyticus* was 75% (30/40), while the total prevalence of *V. vulnificus* was 12.5% (5/40). *A. hydrophila* was identified from 25% (10/40) of total fish examined. Other bacterial spp. identified with few number were, *Streptococcus* spp., *Staphylococcus* spp., *Klebsiella oxytoca* and *Enterobacter* spp.

The colonies of suspected *V. alginolyticus* were rounded, with regular edges, creamy in color and in some cases adhered strongly to the culture media. All strains were Gram-negative, motile curved rods, cytochrome oxidase and catalase positive. The biochemical and physiological characteristics of all isolates were similar and allowed the presumed identification of the bacteria as *V. alginolyticus* with codes 5452744, 5452644 and 7476644 (Table 2).

Table 1: Physicochemical characteristics of the water sample

Parameter	Value
pH (mg/l)	9.97
O_2 (mg/l)	3.9
Temp	23.0
Salinity (g/l)	4.7
EC(dSm^{-1})	2.98
Total salts (mg/l)	1907.2
NH_4 (mg/l)	9.0
NO_3 (mg/l)	5.9
Fe (mg/l)	2.1

Table 2: Biochemical characteristics of the isolated *V. alginolyticus* strains

Nitrate reduction (NO3)	Indole production (TRP)	Glucose fermentation (GLU)	Arginine dihydrolase (ADH)	Urease (URE)	Esculine hydrolysis (ESC)	Gelatin hydrolysis (GEL)	Para-NitroPhenyl-BD-	D-Glucose assimilation (GLU)	L-arabinose assimilation (ARA)	D-mannose assimilation (MNE)	D-mannitol assimilation (MAN)	N-acetyl-glucosamine assimilation	D-maltose assimilation (MAL)	potassium gluconate assimilation	capric acid assimilation (CAP)	adipic acid assimilation (ADI)	malic acid assimilation (MLT)	trisodium citrate assimilation (CIT)	phenylacetic acid assimilation (PAC)	Cytochrome oxidase (OX)
+	V	+	-	-	+	+	V	+	-	+	V	V	+	+	-	-	+	-	-	+

(+) Positive, (-) negative, (V) variable

The molecular identification results using direct sequencing of 16S ribosomal DNA gene confirmed the biochemical identification, gene sequence of the *V. vulnificus* isolate was deposited in Genebank under accession number KU363618.

Pathogenicity Assays: The pathogenicity assay revealed that *V. alginolyticus* was pathogenic to *O. niloticus* fish at $LD_{50}1x10^6$. The experimentally infected fish showed skin depigmentation, off food and ulcerated mouth. The observed PM lesions were ascetic fluids in the intestine and congestion of the visceral organs. *V. alginolyticus* could be re-isolated in pure culture from the experimentally infected fish by the end of observation time (14 days).

Antimicrobial Susceptibility: Antimicrobial susceptibility of *V. alginolyticus* strains showed that *V. alginolyticus* was sensitive to ciprofloxacin, chloramphenicol, Kanamycin, novobiocin, oxytetracycline, streptomycin, nalidixic acid, cefotaxime and vibriostat (O/129), while resistant to ampicillin and amoxycillin. Moderate sensitivities against trimethoprim–sulfamethoxazole and erythromycin were reported.

DISCUSSION

In this study, bacteriological analysis of the examined fishes resulted in the isolation of *V. alginolyticus* and *V. vulnificus* from 87.5% and 12.5% respectively. Although, all fish samples collected appeared to be healthy and no fish morbidity nor mortality were documented in the farm. This could be deduced that bacterial loads in the farm water and sediment was low and could be well tolerated by tilapia.

Coincidence with this result, several studies reported isolation of *V. vulnificus* from tilapia worldwide. *V. vulnificus* biotype 2 strains are pathogens of eel aquaculture [15]. Also, *V. vulnificus* isolated from a moribund tilapia collected in Taiwan [7] and Bangladesh [6].

V. alginolyticus is considered one of the most dangerous pathogens in marine aquaculture causing severe losses among a large numbers of fish and shellfish species [3, 4]. *V. alginolyticus* has been reported in many cases from Egypt. In an Egyptian study, the total prevalence of *V. alginolyticus* was 82.19% and 87.28% for seabream and seabass from the period of February 2013 through August 2013, from some Egyptian coastal provinces [10]. Moreover, *V. alginolyticus* been isolated from Qarun Lake and Suez Gulf with a parentage of (16.73%) of the recorded cases [16].

Similar to this finding, *V. alginolyticus* and *V. vulnificus* were isolated from water, sediment and gills of brackish (salinity 7) water *O. niloticus* cultured in earthen ponds in the Philippines with a total percentage of 1.1% and (5.1%) for *V. Alginolyticus* and *V. vulnificus* respectively [1]. Also, *V. vulnificus* and *V. alginolyticus* were isolated from 2 and 3 naturally infected life fresh water *O. niloticus* fish in Saudi Arabia respectively [17]. Additionally, 11 strains of *V. alginolyticus*, 13 strains of *V. vulnificus* and 12 strains of *A. hydrophila* was isolated from tilapia (*O. niloticus*) fishes of Maga Lake in Cameroon [18].

The lake Qarun is the largest reservoir of agricultural waste water drainage of Fayoum province and its salinity range from 29.2-35.4‰ in winter, 27.1-36.2‰ in spring, 29.3-38.0‰ in summer and 29.7-35.4‰ in autumn [19]. These were considered as enriching factors to the uprising prevalence of *V. alginolyticus* and other zoonotic vibrios. Establishment of fish farms around the lake and using water drainage and irrigation increased the salinity and could allow the transmission of pathogenic bacteria.

Due to the economical importance of cultured *Nile tilapia*, the sharp increase in the ammonia level, water pH, physical contact, sharp decrease in the dissolved oxygen, high water salinity and temperature and ability to scavenge iron are the most possible triggering factors for initiation, establishment and spread of bacterial infection.

These bacteria may rapidly proliferate in tilapia and inadvertently propel the occurrence of disease epizootics. These factors have direct impact on adaptive, innate and cellular immune responses of the fish [10, 20-22].

CONCLUSION

The mortality of fish and the manifested clinical signs were due to vibriosis, mainly caused by *V. alginolyticus*. These bacteria identified in this study are opportunistic pathogens that may be inadvertently under stress conditions of changing water physicochemical parameters and intensive culture lead to disease epizootics. *V. alginolyticus* was pathogenic to *O. niloticus* fish at LD_{50} $1x10^6$. Although, there was no mortality documented in the farm because bacterial loads in the farm water and sediment was low and could be tolerated by tilapia. So, further and regular studies should be conducted to study the bacterial communities from water, sediment and fish reared in fish farms around Qarun Lake.

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