Prevalence of Pathogenic *Vibrio anguillarum* Among *Oreochromis niloticus* Fish Fingerlings Infected with Saprolegniasis Around Qarun Lake

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*Vibrio anguillarum* causes severe economic losses in fish and shellfish species and poses public health concerns. This study aimed to provide some information about the prevalence and virulence determinants of *V. anguillarum* isolated from the farmed *Oreochromis niloticus* fish fingerlings nearby Qarun Lake, El-Fayoum Governorate, Egypt. PCR targeting the rpoA (RNA polymerase alpha subunit) gene was done for confirmation of *Vibrio* spp. Moreover, *V. anguillarum* isolates were biochemically identified and confirmed by 16S rRNA gene sequence. *V. anguillarum* isolates produced caseinase, gelatinase, lipase, lecithinase, haemolysins, and possessed biofilm formation. The virulence-related genes; *empA*, *vah1*, *vah3* and *vah4*, *flaA*, *angM*, and *angR* were detected in all tested isolates. Therefore, proper identification of *V. anguillarum* is crucial to better understand the ecology and distribution patterns of such pathogen within Egyptian aquaculture. Moreover, the water quality in these farms near Qarun Lake was less supportive for larval rearing activities of *O. niloticus* fish.

**Keywords:** *Vibrio anguillarum*, Virulence genes, *Oreochromis niloticus* fingerlings, Egypt.

**Introduction**

The lake Qarun is the largest reservoir of agricultural wastewater drainage of El-Fayoum province, and its mean salinity reached 38‰, the high salinity is a result of the irrigation of the nearby farms from the lake. These were considered as enriching factors to the upsurging prevalence of zoonotic *Vibrio* species [1].

*Vibrio anguillarum* is a pathogenic Gram-negative bacterium that causes high mortalities and severe economic losses in warm- and cold-water fish and shellfish species. The most suitable temperature for *V. anguillarum* to cause vibriosis outbreaks ranged from 5°C to 18°C [2]. While in a cold country like Norway, *V. anguillarum* can cause diseases at water temperatures less than 4°C [3]. *V. anguillarum* are serotyped to 23 O serotypes (O1–O23). Serotypes O1, O2, and O3, are associated with vibriosis in fish [4].

Several virulence-related factors have been reported to be associated with the pathogenesis of *V. anguillarum*, including quorum sensing, iron-uptake mechanisms, lipopolysaccharides, extracellular metalloprotease, proteases, haemolysins, dermatoxin, haemaglutinin, cytotoxin, and a siderophore mediated plasmid-encoded system [2, 5-8].

The predisposing factors inducing vibriosis include bad water quality, pollution, high temperatures, osmotic changes, periods of fish nutrient deprivation, inadequate diet composition, and high population density [2]. These environmental stressors could affect the expression of virulence genes that are involved in the pathogenicity of *V. anguillarum* [9]. Saprolegniasis is responsible for significant economic losses in hatcheries and freshwater fish farms across the globe. Saprolegniasis...
usually appears either as a primary pathogen or as a secondary infection to viral, bacterial, and parasitic agents [10].

The prevalence of pathogenic Vibriospp. amongst economic fish in Egypt possess public health concerns. The availability of methods to correctly identify the Vibriospp. is crucial to understand the ecology and distribution patterns of these microorganisms. Therefore, this study aimed to provide a piece of adequate information about the prevalence and virulence determinants of V. anguillarum isolated from farmed O. niloticus fish larval stages in fish farms around Qarun Lake, El-Fayoum governorate, Egypt.

**Materials and Methods**

**Sampling**

During a winter season outbreak of Saprolegniasis affecting O. niloticus fish fingerlings (January and February 2020), samples were freshly captured from a private fish farm (29°26’37.2"N 30°41’38.4"E) in the vicinity of Qarun Lake, El-Fayoum governorate, Egypt. The whole fish were transported in an ice-cooled insulated box to the laboratory within few hours, where they were investigated.

**Ethical statement**

Animal handling was performed according to the experimental protocol, which was approved by the Animal Ethics Committee of National Research Centre, Dokki, Giza, Egypt, (Ethical approval certificate no. 19158).

**Bacterial identification**

The bacterial strains were isolated from the posterior kidney of the diseased fish on TCBS cholera agar media (Oxoid, UK) and incubated at 25°C for 48 h. The suspected Vibrio strains were subjected to oxidase, catalase, and sensitivity to Vibrio-static reagent O/129. The isolates were then biochemically identified using API20NE strips (BioMerieux, France). Furthermore, the bacterial motility was evaluated on 0.4% nutrient agar supplemented with 8% gelatin powder flooded with saturated ammonium sulfate (gelatinase test), 1% Tween-80 containing (0.015 % wt/v) 80 mM CaCl₂ (lipase test), 10% skimmed milk (caseinase test), 10% egg yolk (lecithinase test), and 5% O. niloticus fish blood (β-hemolytic activity).

**Molecular identification**

Total bacterial DNA was extracted using Purelink™ Genomic DNA Purification Kit (Invitrogen, USA) according to the suggested protocol for Gram-negative bacteria. To confirm Vibriospecies identification, PCR targeting the rpoA (RNA polymerase alpha subunit) gene was done for suspected isolated colonies using two VrpoA primers, as shown in Table (1). Moreover, the bacterium-specific 16S rRNA was amplified using the eubacterial universal pair of primers (63f and 1387r) [11]. The primers were synthesized by (Invitrogen) and listed in Table (1). The PCR conditions were adjusted for 40 cycles using 2× DreamTaQ PCR mastermix (Thermo Scientific, U.K.). The amplified fragment of the 16SrRNA gene was purified using GeneJET Gel purification kit (Thermo Scientific). The purified PCR products were sent for sequencing using Sanger DNA sequencer, Applied Biosystems in both directions using the same primer pairs. BLAST in the NCBI was used for verifying the amplified products. The genomic sequence of V. anguillarum 16S rRNA gene was deposited in the NCBI GenBank.

**Anti-microbial susceptibility**

The anti-microbial susceptibility assay was done as described by CLSI [12] using the disc diffusion method. The antibiotics used were; amoxicillin (25 μg), oxytetracycline (30 μg), erythromycin (15 μg), kanamycin (30 μg), novobiocin (30 μg), chloromphenicol (30 μg), nalidixic acid (30 μg) and cefotaxime (30 μg).

**Determination of extracellular enzymes**

The presence of gelatinase, lipase, caseinase, lecithinase, and β-hemolytic activity of isolated bacteria was determined by streaking onto nutrient agar (Oxoid) supplemented with 8% gelatin powder flooded with saturated ammonium sulfate (gelatinase test), 1% Tween-80 containing (0.015 % wt/v) 80 mM CaCl₂ (lipase test), 10% skimmed milk (caseinase test), 10% egg yolk (lecithinase test), and 5% O. niloticus fish blood (β-hemolytic activity).

**Biofilm production**

Biofilm formation was performed as described previously [13]. The overnight bacterial cultures were diluted 100 times, then 100 μL were incubated in 96-well plates at 25°C for 24 h. The well liquid was discarded and washed thoroughly with distilled water. A total volume of 125 μL of 0.1% crystal violet solution was added to each well. The plates were incubated at room temperature for 15 min and then washed thoroughly with distilled water. The wells were left overnight to dry, then the status was assessed.
Experimental infection
To evaluate the pathogenicity, the LD_{50} was determined for two selected V. anguillarum isolates. Experimental infection was conducted inapparently healthy O.niloticus (weight 50±10g). After acclimatization for two weeks, fish were intraperitoneally injected with 0.1mL of serially diluted bacterial suspensions at a concentration of×10^5 to ×10^8 CFU/fish. The control group was injected with sterile 0.85 % NaCl solution. Fish mortality was recorded for 7 days. Re-isolation of pathogenic V. anguillarum isolates were performed on TCBS agar plates from the posterior kidney of dead fish.

Detection of virulence-related genes
V. anguillarum isolates were subjected to PCR assays to detect the virulence-related genes, including structural genes of the zinc metalloprotease gene (empA), the haemolysin genes (vah1, vah3, and vah4), the flagellum (flaA), and trans-acting transcriptional activator (angM, angR). The specific primers used are shown in Table 1.

Histopathological examination
After complete necropsy of the fish, fresh hepatopancreatic, renal and splenic tissue specimens were collected, fixed in Davidson’s fixative, dehydrated in ascending grades of alcohol, cleared with xylene, paraffin-embedded, sectioned by microtome at 5 µm thickness, stained by H & E and microscopically examined [19].

**TABLE 1. The used primers in identification of V. anguillarum.**

| Target genes | Oligonucleotides sequences | Products (bp) | References |
|--------------|-----------------------------|---------------|------------|
| 16S rRNA     | 63f-CAGGCCTAAACATGCAAGTC    | 1300          | [14]       |
|              | 1387r-GGGCGGWGTGTACAAGGC    |               |            |
|              | F:AAATCGAGCTGGGACCCT        | 456           | [15]       |
|              | R:GCAATTTT(A/G)TC(A/G/T)AC(C/T)GG | 242         |            |
| VrpoA        | F:AAATCGAGCTGGGACCCT        | 524           | [16]       |
|              | R:GTAICGACTTTTGTACGCTGAC    |               |            |
| empA         | F: CTTTTAACAAAGTGGGCTA      | 248           | [17]       |
|              | R: CGATTGTGAAAGGCACAAAT     |               |            |
| vah1         | F: TGCCTATATGTGCTATTACGTT   | 493           | [7]        |
|              | R: GCACCCGTTGTATCATCATCTAAG |               |            |
| vah3         | F: ATGACTTCTCTCTAAATTTCTGTTGTCG | 1128       | [7]        |
|              | R: GATAGAGCCGACCTGTTGCTG    |               |            |
| vah4         | F: ATGAAAACCATAGGCTCAAGATCT | 603           | [7]        |
|              | R: TCACGCTTTTTGGTTATATGAC    |               |            |
| flaA         | F: GTGTGATGACTTCCGTATGG     | 435           | [17]       |
|              | R: GCTCTGCGTGTGTCGTAATC     |               |            |
| angM         | F: TGAAGTGAGGCTGTGTAAG      | 453           | [17]       |
|              | R: TCACGCGGCTTTTTGGTTATATG  |               |            |
| angR         | F: AAGATGAGCCGGAATGCCTGAG   | 957           | [18]       |
|              | R: CTCCGAATCCATAACGATGA     |               |            |
Results

Bacterial identification

The colonies were yellowish color on TCBS medium with a range of (2-3 mm in diameter) with yellow pigmentation. All bacterial isolates were short straight and curved bacilli, Gram-negative, motile, oxidase, and catalase-positive, sensitive to Vibrio-static reagent O/129. All bacterial isolates were indol positive, reduced nitrate to nitrite, fermented lactose, mannitol, sucrose, and glucose without gas production. Further biochemical identification tests using the API20NE confirmed them to be V. anguillarum.

Molecular identification

The primers used to identify Vibrio isolates generated two bacterium-specific fragments of 242-bp and 456-bp amplicons (Fig. 1A). Moreover, PCR amplification using primers VrpoA-F/VrpoA-R generated 524-bp DNA fragments for Vibrio species without non-specific bands (Fig. 1B). V. anguillarum was confirmed by the 16S rRNA gene sequences and submitted to the GenBank database under accession number MW559550. Using NCBI Blast produced 100% homology with other V. anguillarum sequences in the GenBank database.

Anti-microbial susceptibility

The in-vitro antibiotic susceptibility test results revealed that V. anguillarum isolates tested were susceptible to amoxicillin, oxytetracycline, erythromycin, novobiocin, chloramphenicol, nalidixic acid, and resistant to kanamycin and cefotaxime.

Determination of extracellular enzymes

The results of extracellular enzymes production showed that V. anguillarum produces lecithinase (Fig. 2A), caseinase (Fig. 2B), gelatinase, lipase on nutrient agar plates, and large clear β-hemolysis on fish blood agar.

Biofilm production

The biofilm formation results showed that V. anguillarum strains are motile organisms and formed a biofilm at the air-liquid interface.

Experimental infection

The result of the experimental challenge of O. niloticus by V. anguillarum intraperitoneal injection revealed that the LD₅₀ values of two selected isolates of V. anguillarum were 2.7×10⁷ and 6.4×10⁷ CFU/fish, respectively. No mortality was observed in the control group. The infected fish exhibited typical clinical signs of lethargy, skin depigmentation, exophthalmia, corneal opacity, and generalized hemorrhagic septicemia.

Fig. 1. Agarose gel electrophoresis showing the PCR identification of Vibrio spp. (A) PCR amplification of 242-bp and 456-bp amplicons using specific rpoA primers. (B) PCR amplification of 524-bp using primers VrpoA-F/VrpoA-R. Lane 8 in both pictures: 100 bp molecular marker.
Detection of virulence-related genes

Several studies indicated that *V. anguillarum* isolates could carry multiple virulence determinants, which play an important role in the pathogenesis. The PCR profiles of the amplified virulence genes are illustrated in (Fig. 3). The results showed that *empA* (248 bp), *vah1* (493 bp), *vah3* (1128 bp), *vah4* (603 bp), *flaA* (435 bp), *angM* (453 bp), and *angR* (957 bp) genes were detected in all isolates.

Histopathological findings

The histopathological examination of the experimentally infected fishes using *V. anguillarum* revealed the following changes. Hepatopancreas showed severe congestion of hepatic main blood vessels and sinusoidal spaces, with diffuse hepatocytic degenerative changes (Fig. 4a). In spleen, marked congestion and hemorrhages with moderate activation of melano-macrophage centers in the vicinity of necrotic spaces, were also noticed (Fig. 4b). Furthermore, in posterior kidney, focal renal tubular and glomerular as well as interstitial necrotic changes were also evident (Fig. 4c).

Fig. 2. (A) Lecithinase activity of *V. anguillarum* on nutrient agar supplemented with 10% egg yolk. (B) Caseinase activity of *V. anguillarum* on nutrient agar supplemented with 10% skimmed milk indicated by clearing around the colony growth.

Fig. 3. Gel electrophoresis showing the virulence genes of *V. anguillarum*. Lane 2: *vah3* (1128bp), Lane 3, *angR* (957bp), lane 4: *vah4* (603bp), lane 5: *vah1* (493bp), lane 6: *empA* (248bp), lane 7: *flaA* (435bp), Lane 1 and 8: 100bp molecular marker.
Fig. 4. Histopathological findings in the tissues of *O. niloticus* fish infected with *V. anguillarum* after 48 hours (a) Hepatopancreas showing severe congestion of hepatic main blood vessels and sinusoidal spaces, with diffuse hepatocytic degenerative changes. (b) Spleen showing congestion and hemorrhages with moderate activation of melanomacrophage centers in the vicinity of necrotic spaces. (c) Posterior kidney showing focal renal tubular, glomerular and interstitial necrotic changes. H&E staining, Bar = 50 µm.

Discussion

Aquaculture represents an important sector in the Egyptian economy providing essential food facing the growing demand for animal protein. Lake Qarun is an inland closed lake acts as a reservoir for large amounts of polluted agricultural and municipal drainage water of El-Fayoum Governorate. It receives its water from two main drains, EL-Bats and El-Wadi, and loses water by evaporation only with 38‰ salinity [20]. In general aquaculture is subjected to a variable number of ecological stressors, particularly bacterial infection with *Vibrio* spp. *Vibriosis* is a globally threatening bacterial infection widely distributed in the marine- and brackish-water fishes causing high mortalities and severe economic losses. The disease outbreaks occur when fish are sharply exposed to stress factors [2]. Moreover, the adverse aquatic environment and damage caused by *Vibrio* spp. boosted susceptibility of fish to the infection with *Saprolegnia* spp., which is considered a devastating disease in hatcheries and farms worldwide [21].

Dalmasso, La Neve [15] developed a rapid and reliable PCR targeting the *rpoA* gene to identify the genus *Vibrio* without the need for further biochemical tests. Additionally, a multiplex (mPCR) was developed by Zhang, Zhang [16] to detect four pathogenic fish bacteria, including the *rpoA* gene for *Vibrio* species.

In this study, the *Vibrio* species isolated from *Saprolegniasis* was *V. anguillarum* based on its phenotypic characteristics and 16S rRNA sequence analysis. In a previous study, other *Vibrio* spp., namely, *V. alginolyticus* and *V. vulnificus* were isolated from *O. niloticus* cultured in a private farm nearby Qarun Lake [1].

In this study, *V. anguillarum* isolates were positive for the production of caseinase, gelatinase, lipase, lecithinase, and hemolysis. Invasion and pathogenesis of *Vibrio* species depend mainly on bacterial motility, attachment, siderophore-mediated iron-sequestering system, and production of extracellular enzymes as hemolysins, metalloprotease, proteases, dermatotoxin, hemaglutinin, cytotoxin, caseinase, gelatinase, and lipase [2, 8]. These factors are claimed for the pathogenesis of *V. Anguillarum* in the target fish, the clinical signs, postmortem and histopathological picture in this study are a proof of their destructive effect on fish tissues. Similar pathological profile was evident in a previous study [22].

Several studies indicated that *V. anguillarum* isolates could carry multiple virulence genes, which indeed play an important role in the pathogenesis. The PCR confirmed the presence of *empA*, *vah1*, *vah3*, *vah4*, *flaA*, *angM*, *angR* genes in all tested isolates. Using the same primers, Gao, Pi [17] amplified *empA*, *vah1*, *vah2*, *vah3*, *vah4*, *rtxA*, *flaA*, but not *angM*, *angR* genes from stressed starved *V. anguillarum* isolate for six months.
The flagellinA, one of the five-flagellin subunits identified in *V. anguillarum*, is required for efficient invasion of rainbow trout fish [23]. Furthermore, removing the conserved C terminus of flagellin A resulted in a decreased virulence of approximately three logs when fish were infected intraperitoneally and decreased motility by 50% [24].

Norqvist, Normman [25]identified an extracellular metalloprotease (*empA*) with mucinase activity. This protease revealed homology to the elastase of *Pseudomonas aeruginosa* and the protease of *Legionella pneumophila*. This protease help *V. anguillarum* colonize and invade the fish and subsequently cause generalized septicemia. The mutated *empA* is involved in the virulence of *V. anguillarum* both using immersion and intraperitoneal injection[5]. Moreover, recombinant *empA* displayed cytotoxicity to flounder gill cells, necrosis, hemorrhage in the peritoneal cavity, and death for turbot [26]. Similarly, *empA*s identified in other pathogenic *Vibrio* species contributed to their pathogenicity in fish and shellfish larvae [27].

Extracellular haemolysins are important virulence factors for *V. anguillarum*. The first haemolysin, *vah*1 gene, was identified by Hirono, Masuda [28] and showed strong hemolytic activity to carp and rainbow trout erythrocytes. Four other hemolysigenenes (*vah*2, *vah*3, *vah*4, and *vah*5) caused haemolysis on rainbow trout erythrocytes[7]. The haemolytic activity is governed by not just the *vah* genes but by varied and complicated groups of genes such as a putative phospholipase (*plp*), a putative lasonizing lipase (*llpA*), and a repeat in toxin(*rtc*)genes [8]. Similar to our results, Rodkhum, Hirono [7] investigated 70 *Vanguillarum* isolates mostly obtained in Japan, found *vah* 1-5 prevalence as87.14; 100; 98.57; 55.71; and 42.86%, respectively. While the prevalence rates of these genes were 72.54; 100; 98.57; 55.71; and 42.86%, respectively. While the waters in these farms near Qarun Lake were less supportive for *O. niloticus* fish fingerlings hatchery and stocking activities.

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**Conflict of interest**

The authors of this study declare no conflict of interest.

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انتشار بكتيريا فيبريو أنجويللارام الممرضة بين أض无障碍 أسماك البلطي النيلي
(أوريوكروميس نيلوتيكاس) المصابة بالسباريوما حول بحيرة قارون.

عبد الجيد متولي يونس - الخليل سيدي جعفر - علاء الدين زكريا أبو بريكة - وليلي علي محمد
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تسبب بكتيريا الفيبريو أنجويللارام خسائر اقتصادية شديدة في العديد من أنواع الأسماك والمحاريات وقد تثير مخاوف من صحة العامة. تهدف هذه الدراسة إلى تقديم بعض المعلومات حول أسباب انتشار هذه البكتيريا في زريعة أسماك البلطي النيلي (Oreochromis niloticus) في بحيرة قارون، محافظة الفيوم، مصر. تم تحديد جينات البكتيريا على إنه فيبريو بإستخدام جينات 16S بواسطة تفاعل البلمرة المتسلسل (PCR). و تم تأكيد أنها نوع أنجويللارام بواسطة تتابع جين rpoA و بالخصائص البيوكيميائية. و قد وجد أنها تنتج إنزيمات الكازيناز، مواجات، التحليلات، الليفوفيناز، vah1، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU، empV، empW، empX، empY، empZ، empA، empB، empC، vah2، vah3، empR، empS، empT، empU，