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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 *Oreochromisniloticus* fish fingerlings nearby Qarun Lake, El-Fayoum Governorate, Egypt. PCRtargeting the *rpoA* (RNA polymerase alpha subunit)gene was done forconfirmation 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;*emp*A, *vah*1, *vah*3 and *vah*4,*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 agriculturalwastewater 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 theuprising prevalence ofzoonotic*Vibrio* species [1].

Vibrio anguillarum is a pathogenic Gram-negative bacterium thatcauses 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 withthe 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 debrivation, 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

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usually appears either as a primary pathogen or as a secondary infection to viral, bacterial, and parasitic agents [10].

The prevalence of pathogenic *Vibrio*spp. amongt economic fish in Egypt possesspublic health concerns.The availability of methods to correctly identify the *Vibrio*spp. 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-cooledinsulated 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 kidneyof 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 reagentO/129. The isolateswere then biochemically identified using API20NEstrips (BioMerieux, France). Furthermore, the bacterial motility was evaluated on 0.4% nutrient agar supplemented with 2% NaClas described by O'Toole, et al., [6]. The purified isolates of *V. anguillarum* were stored for further investigationsusing 20 % (v/v) glycerol at –80°C.

Molecular identification

Total bacterial DNA was extracted using Purelink™Genomic DNA Purification Kit (Invitrogen, USA))according to the suggested protocol for Gram-negative bacteria. Toconfirm *Vibrio*species identification, PCRtargeting the *rpoA* (RNA polymerase alpha subunit)gene was done for suspected isolated colonies using two VrpoAprimers, as shown in Table (1). Moreover, the bacterium-specific 16S rRNA was amplified using the eubacterialuniversal pair of primers (63f and 1387r)[11].Theprimers 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.).Theamplified fragment of the 16SrRNA gene was purified using GeneJET Gel purification kit (Thermo Scientific). The purified PCR productswere sent for sequencing using Sanger DNAsequencer, Applied Biosystems in both directions using the same primer pairs. BLAST in the NCBIwas used for verifying the amplifiedproducts.The genomic sequence of *V. anguillarum*16S rRNA gene was deposited in the NCBIGenBank.

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), chloromephnicol (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 determinedby streaking onto nutrientagar (Oxoid) supplemented with 8% gelatin powder flooded with saturated ammonium sulfate(gelatinase test), 1% Tween-80containing (0.015 % wt/v) 80 mM $CaCl_2$ (lipase test), 10% skimmed milk (caseinase test),10% egg yolk (lecithinasetest), 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 platesat 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$\times 10^5$ to $\times 10^8$ CFU/fish. The control group was injected with sterile 0.85 % NaCl solution. Fish mortalitywas recorded for 7 days. Re-isolation of pathogenic *V. anguillarum*isolates were performedon TCBS agar plates from the posterior kidney of dead fish.

Detection of virulence-related genes

V. anguillarum isolates were subjected to PCRassays to detect the virulence-related genes, including structuralgenes of the zinc metalloprotease gene (*emp*A), the haemolysin genes(*vah*1, *vah*3, and *vah*4), the flagellum (*flaA*), and trans-acting transcriptionalactivator (*angM*, *angR*). The specific primers used are shownin 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-CAGGCCTAACACATGCAAGTC 1387r-GGGCGGWGTGTACAAGGC	1300	[14]
VrpoA	F:AAATCAGGCTCGGGCCCT R:GCAATTTT(A/G)TC(A/G/T)AC(C/T)GG	456 242	[15]
VrpoA	F:AAATCAGGCTCGGGCCCT R:GTATCGACTTTGGTACGCTGAGC	524	[16]
empA	F: CCTTTAACCAAGTGGGCGTA R: CGATTTGTAAGGGCGACAAT	248	[17]
vah1	F: TGCGCTATATTGTCGATTTCAGTT R: GCACCCGTTGTATCATCATCTAAG	493	[7]
vah3	F:ATGACTTCTTCTAAATTTTCGTTATGTGCG R: GATAGAGCGGACTTTGCTTG	1128	[7]
vah4	F: ATGAAAACCATACGCTCAGCATCT R:TCACGCTTGTTTTTGGTTTAAATGAAATCG	603	[7]
flaA	F: GTGCTGATGACTTCCGTATGG R: GCTCTGCCCGTTGTGAATC	435	[17]
angM	F: TGAAGTTGAGCCTCGTAA R: TCAGACCTGTTGATTCGT	453	[17]
angR	F: AAGAGTGAGCCAATGCGTAG R: CTCCGAATCCATAACGATGA	957	[18]

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 wereshort 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, ferment lactose, mannitol, sucrose, and glucose without gas production. Further biochemical identification tests using the API20NE confirmed them to be *V. anguillarum*.

Molecular identification

The*rpoA* 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 524bpDNA 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 NCBIblast produced100% 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* produceslecithinase(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 LD_{50}values of two selected isolates of *V. anguillarum*were 2.7×10^7 and 6.4×10^7 CFU/fish, respectively. No mortality was observed in the control group. The infected fish exhibited typical clinical signs of lethargy,skin depigmentation, exophthalmia, cornealopacity, and generalized hemorrhagic septicemia.

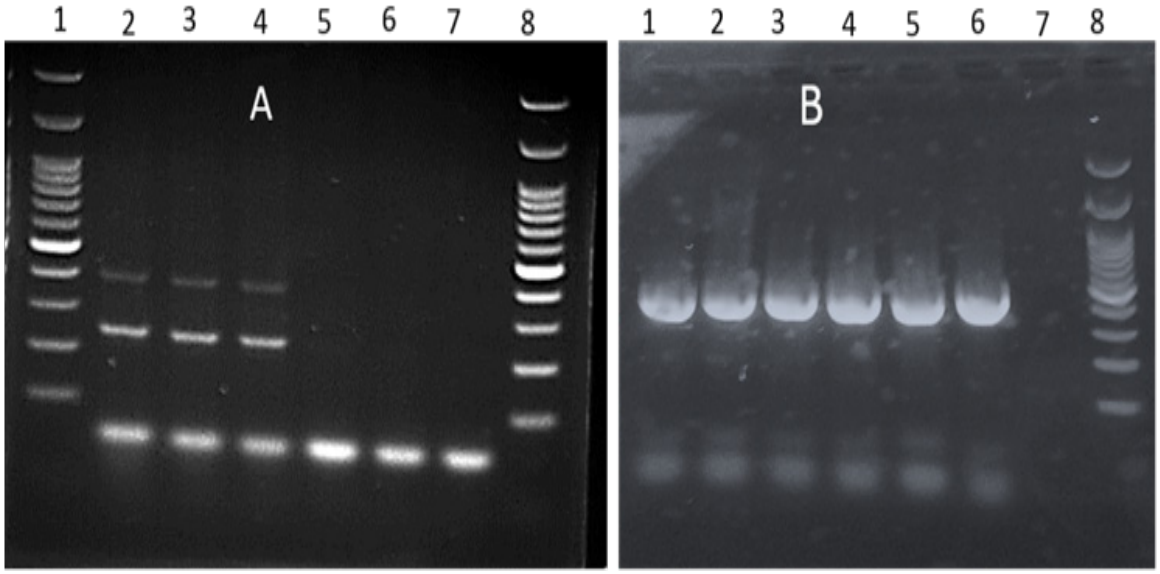

Fig. 1. Agarose gel electrophoresis showing the PCR identification of *Vibrio* spp. (A) PCR amplification of 242-bp and 456-bp amplicons using specific *rpoA* primers. (B) PCR amplification of 524bp using primers VrpoA-F/VrpoA-R. Lane 8 in both pictures: 100 bp molecular marker.

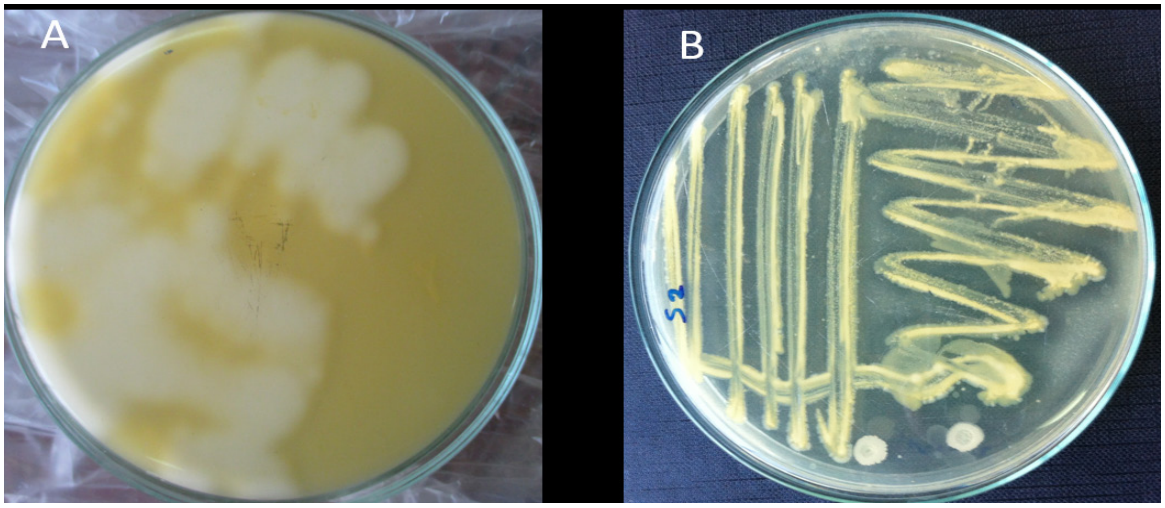

Fig. 2. (A) Lecithinase activity of *V. anguillarum* on nutrient agar supplemented with 10% egg yolk. (B) Caseinase activity of *V. anguillarum*on nutrient agar supplemented with 10% skimmed milk indicated by clearing around the colony growth.

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 showedthat *emp*A (248 bp), *vah*1 (493 bp), *vah*3 (1128 bp), *vah*4 (603 bp), *fla*A (435 bp), *ang*M (453 bp), and*ang*R (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).

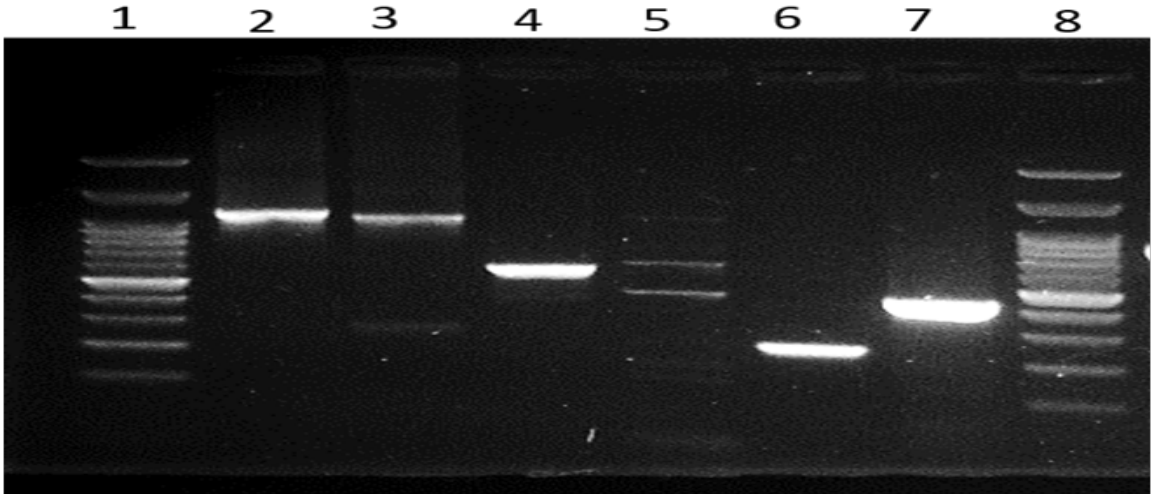

Fig. 3. Gel electrophoresis showing the virulence genes of *V. anguillarum*. Lane 2: *vah3* (1128bp). Lane 3, *angR* (957bp), lane 4: *vah4* (603bp), lane 5: *vah1* (493bp), lane 6: *empA* (248bp), lane 7: *flaA* (435bp). Lane 1 and 8: 100bp molecular marker.

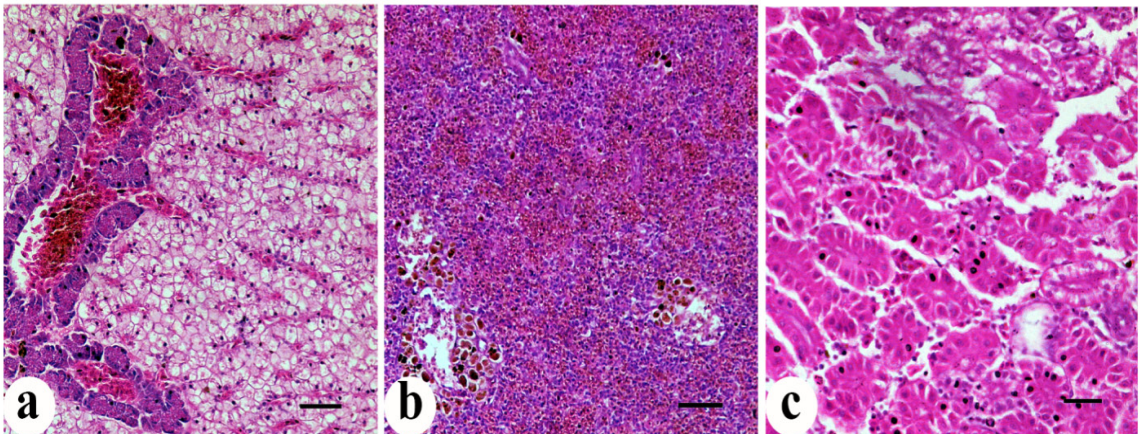

Fig. 4. Histopathological findings in the tissues of *O. niloticus* fish infected with *V. anguillarum* after 48 hours (a) Hepatopancreas showing severe congestion of hepatic main blood vessels and sinusoidal spaces, with diffuse hepatocytic degenerative changes. (b) Spleen showing congestion and hemorrhages 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 facingthe 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 toa variable number of ecological stressors, particularly bacterial infection with *Vibrio* spp.Vibriosis is a globally threatening bacterial infection widelydistributed in the marine- and brackish-water fishes causing high mortalities and severe economic losses. The disease outbreaks occur whenfish are sharplyexposed 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 furtherbiochemical tests. Additionally, a multiplex (mPCR) was developed by Zhang, Zhang [16] todetect four pathogenic fishbacteria, including the *rpoA* gene for *Vibrio* species.

In this study, the *Vibrio* species isolated from *O. niloticus* fish fingerlings that suffered 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 ashemolysins, 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, whichindeed play an important role in the pathogenesis. The PCR confirmed the presence of *emp*A, *vah*1, *vah*3, *vah*4, *fla*A, *ang*M, *ang* Rgenes in all tested isolates. Using the same primers, Gao, Pi [17] amplified *empA*, *vah1*, *vah2*, *vah3*, *vah4*, *vah5*, *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 terminusof flagellin A resulted in a decreased virulence of approximately three logs when fish were infected intraperitoneally and decreased motility by 50 % [24].

Norqvist, Norrman [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].

Extracellularhaemolysins are important virulence factors for *V. anguillarum*. The first haemolysin, *vah*1 gene, was identified byHirono, Masuda [28] and showed strong hemolytic activity to carp and rainbow trout erythrocytes. Four other hemolysingenes (*vah* 2, *vah* 3, *vah* 4, and *vah* 5) causedhaemolysis 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 lactonizing lipase (*llpA*), and a repeat in toxin(*rtx*)genes [8]. Similar to our results, Rodkhum, Hirono [7] investigated 70 *V.anguillarum* 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; 86.27; 82.35; and 17.64 %, respectively, among 51 Turkish *V.anguillarum* isolates [29].

Iron-sequestering systemspermit *Vibrio* species to surviveand cause infection within their host[30].*angM* is involved in the anguibactin biosynthesis. While, *angR*has a crucial role in anguibactin synthesis and regulationof gene expression.These genes have been identified in the virulence plasmid pJM1and clustered in the iron transport biosynthetic operon (ITBO) with the highestexpression level when the iron is limited[18, 31, 32].

Conclusion

Proper identification of *V.anguillarum*is crucial to better understand disease ecology and distribution patterns within Egyptian aquaculture. In this study, *V. anguillarum*isolates produced multiple extracellular enzymes and virulence-related genes, which play an important role in the pathogenesis. *O. niloticus*fish larvae incubated in polluted water during winter are more susceptible to *V.anguillarum and* Saprolegniasis. Thereby, 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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References

1. Younes, A., Fares, M., Gaafar, A. and Mohamed, L.A. Isolation of *Vibrio alginolyticus* and *Vibrio vulnificus* strains from cultured *Oreochromis niloticus* around Qarun Lake, Egypt.*Global Veterinaria*. **16**(1), p. 01-05 (2016).

2. Frans, I., Michiels, C.W., Bossier, P., Willems, K., Lievens, B. and Rediers, H. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention.*Journal of fish diseases*. **34**(9), p. 643-661 (2011). https://doi.org/10.1111/j.1365-2761.2011.01279.x

3. Olafsen, J.A., Christie, M. and Raa, J. Biochemical ecology of psychrotrophic strains of Vibrio anguillarum isolated from outbreaks of vibriosis at low temperature. Zentralblatt für Bakteriologie Mikrobiologie und Hygiene: I. Abt. Originale C: Allgemeine, *angewandte und ökologische Mikrobiologie*. **2**(4), 339-348 (1981). https://doi.org/10.1016/s0721-9571(81)80027-0

4. Toranzo, A., Magariños, B. and Avendaño-Herrera, R. Vibriosis: *Vibrio anguillarum*, *V. ordalii* and *Aliivibrio salmonicida.Fish Viruses and Bacteria: Pathobiology and Protection*. p. 314-333 (2017). https://doi.org/10.1079/9781780647784.0314

5. Milton, D.L., Norqvist, A. and Wolf-Watz, H. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum. Journal of Bacteriology*. **174**(22), 7235-7244 (1992). https://doi.org/10.1128/jb.174.22.7235-7244.1992

6. O'Toole, R., Milton, D.L. and Wolf-Watz, H. Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum.Molecular Microbiology*,**19**(3), 625-637 (1996). https://doi.org/10.1046/j.1365-2958.1996.412927.x

7. Rodkhum, C., Hirono, I., Crosa, J.H. and Aoki, T. Multiplex PCR for simultaneous detection of five virulence hemolysin genes in *Vibrio anguillarum.Journal of Microbiological Methods*. **65**(3), 612-618 (2006). https://doi.org/10.1016/j.mimet.2005.09.009

8. Naka, H. and Crosa, J.H. Genetic determinants of virulence in the marine fish pathogen *Vibrio anguillarum.Fish Pathology*. **46**(1), 1-10 (2011). https://doi.org/10.3147/jsfp.46.1

9. Guanhua, Y., Wang, C., Wang, X., Ma, R., Zheng, H., Liu, Q., Zhang,Y., Ma, Y. and Wang, Q. Complete genome sequence of the marine fish pathogen Vibrio anguillarum and genome-wide transposon mutagenesis analysis of genes essential for *in vivo* infection.*Microbiological Research*. **216**, 97-107 (2018). https://doi.org/10.1016/j.micres.2018.08.011

10. Emara, E.K., Gaafar, A.Y. and Shetaia, Y.M. *In vitro* screening for the antifungal activity of some Egyptian plant extracts against the fish pathogen *Saprolegnia parasitica.Aquaculture Research*. **51**(11), 4461-4470 (2020). https://doi.org/10.1111/are.14791

11. Younes, A.M., Gaafar, A.Y., Abu-Bryka, A. Z., Mohamed, L.A. and Bayoumy, E.M. Genotyping and pathogenicity of *Streptococcus iniae* strains recovered from cultured *Oreochromis niloticus* at Kafr El-Shiekh Governorate, Egypt.*Egyptian Journal of Aquatic Biology and Fisheries*. **23**(3), 467-474 (2019). https://doi.org/10.21608/ejabf.2019.51630

12. CLSI, Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated from Aquatic Animals. (2006).

13. O'Toole, G.A., Microtiter dish biofilm formation assay.*JoVE (Journal of Visualized Experiments)*. (47), e2437 (2011). https://doi.org/10.3791/2437

14. Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J. and Wade, W.G. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA.*Applied and Environmental Microbiology*. **64**(2), 795-799 (1998). https://doi.org/10.1128/aem.64.2.795-799.1998

15. Dalmasso, A., La Neve, F., Suffredini, E., Croci, L., Serracca, L., Bottero, M.T. and Civera, T. Development of a PCR assay targeting the rpoA gene for the screening of *Vibrio* genus.*Food Analytical Methods*. **2**(4), 317-324 (2009). https://doi.org/10.1007/s12161-009-9089-9

16. Zhang, D., Zhang, Q. and Li, A. Development of a multiplex PCR assay for rapid and simultaneous detection of four genera of fish pathogenic bacteria. *Letters in Applied Microbiology*. **59**(5), 471-478 (2014). https://doi.org/10.1111/lam.12303

17. Gao, X., Pi, D., Chen, N., Li, X., Liu, X., Yang, H., Wei, W. and Zhang, X. Survival, virulent characteristics, and transcriptomic analyses of the pathogenic *Vibrio anguillarum* under starvation stress.*Frontiers in Cellular and Infection Microbiology*,**8**, Article no.389, p. 1-12 (2018). https://doi.org/10.3389/fcimb.2018.00389

18. Chen, Q., Wertheimer, A.M., Tolmasky, M.E. and Crosa, J.H. The *Ang*R protein and the siderophore anguibactin positively regulate the expression of iron-transport genes in *Vibrio anguillarum. Molecular Microbiology*,**22**(1), 127-134 (1996). https://doi.org/10.1111/j.1365-2958.1996.tb02662.x

19. Roberts, R.J., Fish pathology. *John Wiley & Sons* (2012). https://doi.org/10.1002/9781118222942

20. Younes, A., NourEldin, A. and Abd Ellatif, M. A contribution of crustacean isopodoa, bacterial infections and physicochemical parameters in mass mortalities among fishes in Lake Qarun. *Research Journal Of Pharmaceutical Biological And Chemical Sciences*,**7**(2), 1906-1911 (2016).

21. Hussein, M.M., Hatai, K. and Nomura, T. Saprolegniosis in salmonids and their eggs in Japan.*Journal of Wildlife Diseases*. **37**(1), 204-207 (2001). https://doi.org/10.7589/0090-3558-37.1.204

22. Mahrous, K.F., Aboelenin, M.M., Abd El-Kader, H.A., Mabrouk, D.M., Gaafar, A.Y., Younes, A.M., Mahmoud, M.A. Khalil,, W.K. and Hassanane, M.S. Piscidin 4: Genetic expression and comparative immunolocalization in Nile tilapia (*Oreochromis niloticus*) following challenge using different local bacterial strains.*Developmental & Comparative Immunology*,**112**, Artcle no. 103777 (2020). https://doi.org/10.1016/j.dci.2020.103777

23. McGee, K., Hörstedt, P. and Milton, D.L. Identification and characterization of additional flagellin genes from *Vibrio anguillarum.Journal of Bacteriology*,**178**(17), 5188-5198 (1996). https://doi.org/10.1128/jb.178.17.5188-5198.1996

24. Milton, D.L., O'Toole, R., Horstedt, P. and Wolf-Watz, H. Flagellin A is essential for the virulence of *Vibrio anguillarum.Journal of Bacteriology*,**178**(5), 1310-1319 (1996). https://doi.org/10.1128/jb.178.5.1310-1319.1996

25. Norqvist, A., Norrman, B. and Wolf-Watz, H. Identification and characterization of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum.Infection and Immunity*. **58**(11), 3731-3736 (1990). https://doi.org/10.1128/iai.58.11.3731-3736.1990

26. Yang, H., Chen, J., Yang, G., Zhang, X.-H., Li, Y. and Wang, M. Characterization and pathogenicity of the zinc metalloprotease *emp*A of *Vibrio anguillarum* expressed in *Escherichia coli. Current Microbiology*. **54**(3), 244-248 (2007). https://doi.org/10.1007/s00284-006-0495-6

27. Hasegawa, H., Gharaibeh, D.N., Lind, E.J. and Häse, C.C. Virulence of metalloproteases produced by Vibrio species on Pacific oyster *Crassostrea gigas* larvae.*Diseases of Aquatic Organisms*. **85**(2), 123-131 (2009). https://doi.org/10.3354/dao02070

28. Hirono, I., Masuda, T. and Aoki, T. Cloning and detection of the hemolysin gene of*Vibrio anguillarum.Microbial pathogenesis*. **21**(3), p. 173-182 (1996). https://doi.org/10.1006/mpat.1996.0052

29. Avsever, M. and Ün, C. Distribution of hemolysin genes in Turkish *Vibrio anguillarum* isolates.*Bull Eur. Ass. Fish Pathol*.,**35**, 75-84 (2015).

30. Tolmasky, M.E., Actis, L. and Crosa, J.H. Genetic analysis of the iron uptake region of the *Vibrio anguillarum* plasmid pJM1: molecular cloning of genetic determinants encoding a novel trans activator of siderophore biosynthesis.*Journal of Bacteriology*. **170**(4), 1913-1919 (1988). https://doi.org/10.1128/jb.170.4.1913-1919.1988

31. Stork, M., Di Lorenzo, M., Welch, T.J., Crosa, L.M. and Crosa, J.H. Plasmid-mediated iron uptake and virulence in *Vibrio anguillarum. Plasmid*. **48**(3),222-228 (2002). https://doi.org/10.1016/s0147-619x(02)00111-7

32. 32. Di Lorenzo, M., Stork, M., Naka, H., Tolmasky, M.E. and Crosa, J.H. Tandem heterocyclization domains in a nonribosomal peptide synthetase essential for siderophore biosynthesis in *Vibrio anguillarum.Biometals*. **21**(6), 635-648 (2008). https://doi.org/10.1007/s10534-008-9149-4.

انتشار بكتيريا فيبريو أنجويللارام الممرضة بين أصبعيات أسماك البلطي النيلي (أوريوكروميس نيلوتيكاس) المصابة بداء السابروليجنيا حول بحيرة قارون.

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تسبب بكتيريا الفيبريو أنجويللارام خسائر اقتصادية شديدة في العديد من أنواع الأسماك والمحاريات وقد تثير مخاوف منتعلقة بالصحة العامة. تهدف هذه الدراسة إلى تقديم بعض المعلومات حول أسباب انتشار وضراوة هذه البكتيريا في زريعة أسماك البلطي النيلي (*Oreochromis niloticus*) المستزرعة بالقرب من بحيرة قارون ، محافظة الفيوم ، مصر. تم تحديد جنس البكتيريا على إنه فيبريو بإستهداف جينات (*rpoA*) بواسطة تفاعل البلمرة المتسلسل (PCR). و تم تأكيد أنها نوع أنجويللارام بواسطة تتابع جين (16S rRNA) و بالخصائص البيوكيميائية. و قد وجد أنها تنتج إنزيمات الكازيناز ، جيلاتيناز ، الليباز ، الليسيثيناز والهيموليسين ، ويمكنها تشكيل البيوفيلم. تم الكشف عن الجينات المرتبطة بالضراوة مثل (empA ، vah1 ، vah3 , vah4 ، flaA ، angM ، و angR) في جميع العزلات التي تم اختبارها.لذلك ، فإن التحديد الصحيح لبكتيريا الفيبريو أنجويللارامأمر بالغ الأهمية لفهم أنماط بيئة والتوزيع لمثل هذه البكتيريا الممرضة داخل المزارع السمكية المصرية. علاوة على ذلك ، كانت جودة المياه في هذه المزارع بالقرب من بحيرة قارون أقل دعما لأنشطة تربية زريعة أسماك البلطي النيلي.

الكلمات الدالة: بكتيريا الفيبريو أنجويللارام ، جينات الضراوة ، زريعة أسماك البلطي النيلي ، مصر.