Pathogenicity, genetic typing, and antibiotic sensitivity of *Vibrio alginolyticus* isolated from *Oreochromis niloticus* and *Tilapia zillii*

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SUMMARY

Although *Vibrio alginolyticus* has been depicted as an omnipresent species in the marine environment, few studies accentuate their incidence in a brackish water system. The present study aimed to investigate the prevalence of *V. alginolyticus* in *Tilapia zillii* and *Oreochromis niloticus* as a comparative study. The genetic and phenotypic characterization, as well as the antibiotic susceptibility of all isolates, were considered. A total of 140 fish, 70 of each species were collected randomly from Temsah lake and brackish water farms at Ismailia governorate, Egypt and were subjected to clinical and bacteriological examinations. Most of the examined fish displayed skin hemorrhages, corneal opacity, and friable liver. The prevalence of *V. alginolyticus* was 48.6% and 35.7% in *T. zillii* and *O. niloticus,* respectively. PCR was performed using sets of primer targeting 16sr RNA gene, where all strains were positive with amplicon size 663 bp. Most of the isolated strains were sensitive to ciprofloxacin and florfenicol while showed high resistance to erythromycin and sulfamethoxazole-trimethoprim. Experimentally infected species with *V. alginoltyicus* showed variable mortalities and proposed a higher susceptibility of marine species. The current study gave insight into the role of water salinity on disease occurrence and emphasized the importance of phenotypic and genotypic characterization in disease diagnosis.

Keywords: Antibiotic resistance, Nile tilapia, PCR, Prevalence, *Vibrio alginolyticus*

RÉSUMÉ

Pathogénicité, typage génétique et sensibilité aux antibiotiques de *Vibrio alginolyticus* isolé à partir d'*Oreochromis niloticus* et de *Tilapia zillii*.

Bien que *Vibrio alginolyticus* ait été décrit comme une espèce omniprésente dans le milieu marin, peu d'études ont présenté leur incidence dans un système d'eau saumâtre. La présente étude visait à étudier la prévalence comparée de *V. alginolyticus* chez *Tilapia zillii* et *Oreochromis niloticus*. La caractérisation génétique et phénotypique, ainsi que la sensibilité aux antibiotiques de tous les isolats, ont été réalisées. Au total, 140 poissons, 70 de chaque espèce, ont été prélevés au hasard dans des exploitations d'eaux saumâtres du lac Temsah et dans le gouvernorat d'Ismaïlia, en Égypte, et ont été soumis à des examens cliniques et bactériologiques. La plupart des poissons examinés présentaient des hémorragies cutanées, une opacité de la cornée et un foie friable. La prévalence de *V. alginolyticus* était respectivement de 48,57% et 35,7% chez *T. zillii* et *O. niloticus*. La PCR a été réalisée en utilisant des ensembles d'amorce ciblant le gène de l'ARN 16sr, où toutes les souches étaient positives avec une taille d'amplicon de 663 pb. La plupart des souches isolées étaient sensibles à la ciprofloxacine et au florfénicol, mais présentaient une résistance élevée à l'érythromycine et au sulfamethoxazole-trimethoprime. Les espèces expérimentalement infectées par *V. alginoltyicus* ont présenté une mortalité variable et une plus grande sensibilité des espèces marines. Cette étude a permis de mieux comprendre le rôle de la salinité de l'eau dans l'apparition de la maladie et a souligné l'importance de la caractérisation phénotypique et génotypique dans le diagnostic de la maladie.

Mots-clés : Résistance aux antibiotiques, tilapia du Nil, PCR, prévalence, *Vibrio alginolyticus*

Introduction

Aquaculture is represented as one of most important functional food-delivering divisions that provide the total populace with a protein of animal origin that compensates the food shortage globally, tilapia is considered the second largest group produced from aquaculture after cyprinids. Among various tilapia species, the Nile tilapia represent the main cultured type due to its economical price, palatability, and the easy cultivation in rivers, pond or dams [32]. Recently, the excessive use of water resources particularly the freshwater ones created several limitations in aquaculture development. Therefore, seawater is the immediate alternative sources for rearing and husbandry of several marine species including *T. zillii*. Aquaculture diseases are the most serious issues confronting fish industries during fish rearing and husbandry. Like different creatures,, fish are subjected to various threaten pathogens, particularly prompted by coexisting of bacterial pathogens [39]. Vibriosis is recognized as one of the most prominent diseases frequently affecting a wide variety of cultured species all over the world [26, 6], and it recognized by specific receptors belong to the pathogen recognition receptor system [3].

V. alginolyticus a ubiquitous organism has been isolated from cultured *O. niloticus* [41], several marine organisms [7, 18], and associated with large scale losses of several fish species [22]. The pliancy of *Vibrio* genomes, besides the recurrent events of horizontal gene transfer [19] revealed some difficulties in the identification of *Vibrio* from the aquatic environment. Hence, recent phenotypic and genomic techniques have been bloomed for rapid identification of

Vibrio spp, not only for its economic importance but also for its public health concern [40]. Polymerase chain reaction (PCR) as a reliable recent technique has the ability to amplify certain DNA molecule among a high concentration of non-target molecules with high specificity and sensitivity [17,14]. The most popular targeting genes are 16S rRNA and housekeeping genes [12]. Although several studies briefly elucidate the incidence of *V. alginolyticus* among different marine species since it was halophilic and strongly correlated with high salinity. Little was known about their pathogenicity, prevalence and molecular characterization in freshwater fishes. Therefore, the current work aimed to investigate the pathogenicity, prevalence as well as the antibiotic sensitivity of *V. alginolyticus* in cultured *O. niloticus* and *T. zillii*. In addition, the genetic typing of the isolated strains was performed using PCR.

Material and methods

FISH SAMPLING

In order to investigate the prevalence of *V. alginolyticus*, 140 fish of two different species (70 *T. zillii* and 70 *O. niloticus*) with average body weight 60± 10 g were collected randomly along the parallel pelagic road of Temsah lake and private brackish water farms, respectively at Ismailia governorate, Egypt, from June to August 2017. Fish specimens were rapidly transferred alive in strong plastic bags supplied with compressed air to the bacteriological lab at the Faculty of Veterinary Medicine, Suez Canal University for further investigations.

CLINICAL AND POSTMORTEM EXAMINATIONS

Fish were examined for any abnormal lesions and internal lesions according to the methods described by Austin and Austin [8].

BACTERIOLOGICAL EXAMINATION

Sampling was carried out under complete aseptic conditions from the internal organs (liver, kidney, spleen and heart from each fish), then the collected samples were inoculated in Tryptic soy broth and incubated at 29 °C for 24 h. A loopful of the incubated broth was streaked on thiosulfate citrate bile salt sucrose agar (TCBS) as well as on blood agar for detection of hemolysis, then the streaked plates were incubated at 29 °C for 24 h. Bacterial isolates were distinguished morphologically utilizing Gram's stain and in addition biochemically utilizing methods depicted by Quinn, Markey [34].

GENETIC TYPING OF *V. ALGINOLYTICUS*

DNA of purified bacteria was extracted using QIAamp DNA Mini Kit (Invitrogen, USA). To verify that the isolated strains are belong to *V. alginolyticus,* two sets of primers targeting 16S rRNA gene were selected according to Tarr, Patel [37]. The oligonucleotide primers used in this study are illustrated in (Table I). PCR reaction mixtures of 50 µl were amplified in MJ Mini™ Gradient Thermocycler apparatus (Biometra) using a commercial kit of Green Master Mix (NZYtech). A reaction with no template DNA used as negative control, while positive controls were references strains kindly supplied by Animal Health Research Institute in Dokki, Cairo, Egypt. All samples were exposed to initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing 50 °C for 45 s, and acquiring the signal at 72 °C for 45 s. 10 min of final extension at 72 °C was adopted. Amplified products were screened by 2% (w/v) agarose gel electrophoresis for 1 h at 80 V in 1X TAE buffer (0.04 M Tris,0.0001 M EDTA, pH 8.0), visualized using 15 µl of DNA gel stain (Sigma-Aldrich) and photographed under UV transilluminator. A 100 bp ladder (SOL BIODYNE) was used as a molecular mass marker.

ANTIBIOTIC SUSCEPTIBILITY TEST

The sensitivity to five commercially available antibacterial agents (Oxoid) including; ciprofloxacin, nalidixic acid, *sulfamethoxazole*-trimethoprim, erythromycin, and florfenicol was performed using a disc diffusion technique. The diameter of inhibition zone was estimated by millimeter and expressed as sensitive, intermediate, and resistant according to National Committee for Clinical Laboratory Standard of Antimicrobial Susceptibility NCCLS [31].

EXPERIMENTAL INFECTION (PATHOGENICITY TEST)

Acclimation period

A total of 40 apparently healthy fish represented as (20 *O. niloticus* weighing 60 ± 10 g and 20 *T. zillii* weighing 40 ± 5) were obtained from Fish Research Center, Suez Canal University and from a private fish farm at Ismailia governorate with no history of disease. The fish have been adapted for 2 weeks into four identical glass units of 100 L holding capacity. The glass units (n= 2) that contribute *O. niloticus* were supplied with chlorine-free freshwater, while others contributing *T. zillii* were supplied with aerated seawater with salinity averaged 30 ± 2 g/L. Dissolved oxygen was maintained at 6 ± 2 mg/L using electrical air pumping compressors (RINA, Italy), while the temperature was adjusted thermostatically at 25 °C ± 1 °C using heaters (Type

Gene	Primer	Sequence (5'–3')	Product length	Reference
16S rRNA	*V.16S-700 F*	CGGTGAAATGCGTAGAGAT	663 bp	**[37]**
	V.16S-1325 R	TTACTAGCGATTCCGAGTTC		

Table I: Oligonucleotide primers used in PCR

CMI, Germany). Water pH was maintained at 7.5 and 12 hours light/12 hours dark photoperiod was embraced. To maintain ammonia level within a permissible limit, the fecal matter and other wastes were siphoned off, and 50% of the water was changed daily meanwhile the trial. The fish were given commercial pellets (Skretting, Egypt) of 30-35% crude protein until visual satiety.

Challenge test

It was performed according to the universal directive on the protection of animals used for scientific purposes. Fish of such species were randomly assigned into two groups each contributes one-glass units of 100 L holding capacity and 10 fish stocking density. Fish of the first group were injected I/P with phosphate buffer saline (negative control), while those of the other group were inoculated with 0.1 ml of *V. alginolyticus* at concentration 1×10^8 CFU/ml according to Younes, Fares [41] and Hussain [23]. Bacterial counts were performed by means of Helber count chamber and confirmed using plate count technique. The cumulative mortalities were noticed for 10 days after the challenge. Dead fish were collected, investigated aseptically to clarify the etiology of death. To ensure Koch's postulates, mortalities were only considered when *V. alginolyticus* was retrieved from challenged fish. Any survivors at the end of the experiments were euthanized, and examined for detection of *V. alginolyticus*. Fish were kept up as per the National and International Institutional Guidelines for the Care and Use of Animals for Scientific purposes and morally endorsed from the supported establishment (Suez Canal University).

Results

CLINICAL EXAMINATION

In the present study, most of the naturally infected fish showed exophthalmia (Fig 1A), corneal opacity and distended abdomen (Fig 1B). Scattered wide irregular hemorrhagic spots mainly at the base of pectoral and anal fins, and at the trunk region were also noticed (Fig 1C).

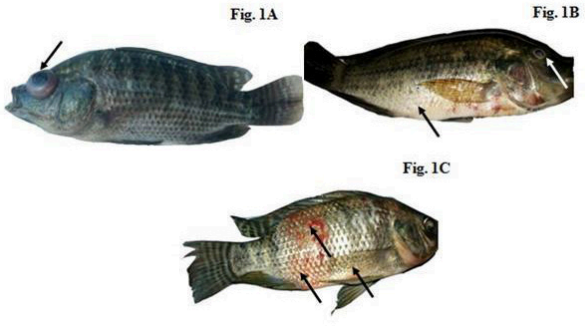

Figure 1: Naturally infected fish with *V. alginolyticus* (A) *O. niloticus* showing exophthalmia (B) *T. zillii* showing corneal opacity and abdominal distension (c) *O. niloticus* showing scattered wide irregular hemorrhagic spots at the base of pectoral and anal fins, and at the trunk region.

POSTMORTEM EXAMINATION

The necropsy finding revealed the presence of hemorrhagic livers, distended gall bladders, engorged spleens, and congested kidneys (Fig 2A). The intestine appeared hemorrhagic and filled with yellow serous fluid (Fig 2B).

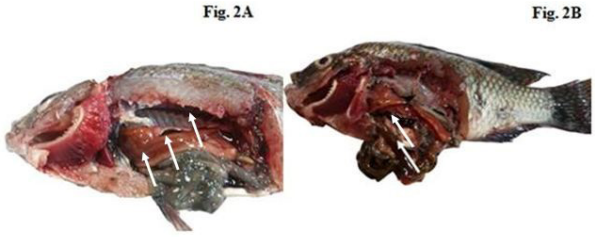

Figure 2: Naturally infected fish with *V. alginolyticus* (A) *O. niloticus* showing hemorrhagic liver, engorged spleen, and congested kidney (B) *T. zillii* showing diffused hemorrhagic intestine with yellow serous fluid.

BACTERIOLOGICAL EXAMINATION

All isolates were Gram-negative, motile curved bacilli grow on tryptic soy agar (TSA) and TCBS media supplemented with 2% NaCl at 29 °C for 24 h. The colonies were circular whitish yellow in color and exhibited a swarming activity on TSA, whereas on TCBS they displayed yellow colored colonies. Biochemically as showed (Table II), all isolates were oxidase and catalase positive, fermentable, sensitive to novobiocin. They were positive in respect to, indole, methyl red, Voges-Proskauer (VP), and citrate utilization test, while they were negative for arginine hydrolysis and hydrogen sulfide (H_2S) production. Their metabolism is aerobic or facultative anaerobic and they ferment carbohydrates with the production of acid but no gas. The results of the bacteriological examination revealed that the prevalence of *V. alginolyticus* was 48.6% and 35.7 % in *T. zillii* and *O. niloticus,* respectively as showed (Table III). The highest prevalence was recorded in liver (39.3%), followed by the kidney (30%), spleen (21.3%), and heart (9.3%) as showed (Table IV).

GENETIC TYPING OF *V. ALGINOLYTICUS*

All retrieved strains were positive for 16S rRNA conserved gene of *V. alginolyticus* with specific amplicon size 663bp as illustrated in (Fig 3).

ANTIBIOTIC SUSCEPTIBILITY TEST

All evaluated strains were highly sensitive to ciprofloxacin (97.3%) and florfenicol (94.7%), moderately sensitive to nalidixic acid (78.7%), and were highly resistant to erythromycin (97.3%) and *sulfamethoxazole*-trimethoprim (72%) as showed (Table V).

Test	*Vibrio alginolyticus*
Gram reaction	-
Motility	+
Shape	Curved rods
Oxidase	+
Catalase	+
Growth on	
TCBS	Yellow colonies
TSA	Whitish yellow
O/F	F
Growth on	
0 % NaCl	-
0.5% NaCl	+
2% NaCl	+
5% NaCl	+
7% NaCl	+
10% NaCl	+
Indole	+
M.R	+
V.P	+
Citrate utilization	+
Arginine hydrolysis	-
H_2S production	-
Novobiocin	S

- (Negative); + (Positive); F (fermentative); S (Sensitive).

Table II: Phenotypic and biochemical characteristics of *V. alginolyticus* strains isolated from *T. zillii* and *O. niloticus*

Fish species	No. of Examined Fish	No. of infected fish with *V. alginolyticus*	Prevalence of *V. alginolyticus* (%)
T. zillii	70	34	48.6
O. niloticus	70	25	35.7
Total	140	59	42.1

Table III: Prevalence of *V. a1ginolyticus* among naturally infected *T. zillii* and *O. niloticus*

Fish species	Total No. of isolates	Prevalence of isolates among different organs							
		Liver		Kidney		Spleen		Heart	
		No	%	No	%	No	%	No	%
T. zillii	85	34	40	26	30.6	17	20	8	9.4
O. niloticus	65	25	38.5	19	29.2	15	23	6	9.2
Total	150	59	39.3	45	30	32	21.3	14	9.3

Table IV: Prevalence of *V. alginolyticus* in different internal organs of naturally infected *T. zillii* and *O. niloticus*

Antimicrobial agents	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ciprofloxacin	146	97.3	4	2.7	-	-
Florfenicol	142	94.7	8	5.3	-	-
Nalidixic acid	-	-	118	78.7	32	21.3
Erythromycin	-	-	4	2.7	146	97.3
Sulfamethoxazole-trimethoprim	-	-	42	28	108	72

Table V: Antimicrobial susceptibility of *V. alginolyticus* strains isolated from naturally infested *T. zillii* and *O. niloticus*

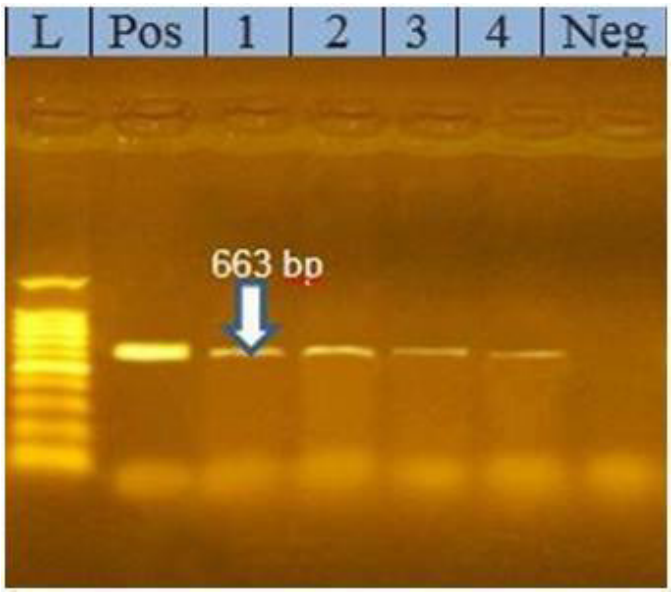

Figure 3: Electrophoretic pattern of 16S rRNA PCR assay of *V. alginolyticus* strains isolated from fish. Lane 1, molecular weight ladder; lane 2, a positive control; lanes 3-5, the specific DNA product amplified from *V. alginolyticus* strains isolated from *T. zillii*; lane 6, the specific DNA product amplified from *V. alginolyticus* strains isolated from *O. niloticus;* lane 7, a negative control.

EXPERIMENTAL INFECTION (PATHOGENICITY TEST)

The cumulative mortality of the experimentally infected fish was evaluated for 10 days after inoculation (Fig 4). Fish injected with physiological saline did not demonstrate any mortalities or pathological lesions, while those inoculated with 0.1 ml of *V. alginolyticus* at concentration of 1×10^8 CFU/ml showed nearly the same clinical signs and postmortem lesions found in naturally infected fish. Experimentally infected *T. zillii* produced higher mortalities in a shorter time than *O. niloticus* under the same conditions. In fact, all infected *T. zillii* died within 5 days post inoculation, while the infected *O. niloticus* dead within 10 days after inoculation.

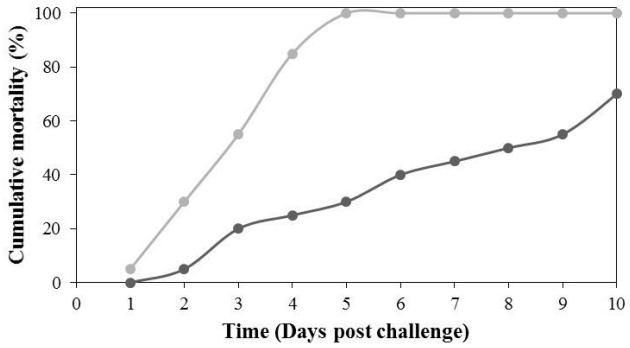

Figure 4: Cumulative mortality of *T. zillii* (●) and *O. niloticus* (●) subjected to intraperitoneal injection with 0.1 ml of *V. alginolyticus* at concentration 1×10^8 CFU/ml.

Discussion

Vibrio alginolyticus is viewed as a standout amongst the most compromising pathogens causing high mortalities and huge economic crises in fish producing sectors worldwide [30]. In the present study, exophthalmia, corneal opacity, distended abdomen, and scattered hemorrhagic spots were the most prominent lesions, in agreement with those observed by [39, 20, 35]. The necropsy findings were nearly similar to those obtained by [20, 36]. Signs of hemorrhagic septicemia observed in the current study were mainly attributed to *V. alginolyticus* serum proteases [13], hemolytic toxins [27-21], or due to the production of multiple virulent extracellular products including proteases, haemolysin, and siderophores which thought to be associated with the virulence of the bacteria [4]. The hydrolytic and haemolytic components of *V. alginolyticus* ECPs were exceedingly poisonous and in charge of the obtrusive and proliferative procedures of the bacteria [9]. The destruction of crucial blood elements induced by *V. alginolyticus* could be the fundamental cause of mortality [27].

Regarding the phenotypic characters of *V. alginolticus*, our results were inconsistency with those reported by [29], and biochemically they were comparable to those obtained by [36, 42]. In the present work, the results of the bacteriological examination revealed that the prevalence of *V. alginolyticus* among examined species were relatively low compared to those reported by [41] who reported that the prevalence of *V. alginolyticus* was 75% in *O. niloticus*. The variation in bacterial prevalence among such population may relate to the differences in the degree of water salinity, host susceptibility, number of samples investigated, and other different environmental conditions.

The prevalence pattern among various species in the current study reflected higher dominance at higher salinity zone, with a noteworthy relationship with the saltiness. Thus, was attributed to the great salt tolerance capacity of *V. alginolyticus* together with osmoregularity [28]. The main predisposing factor in different outbreaks was the elevated water temperature. The adverse effect of the elevated temperature could initiate a stressful situation, which subsequently reduces the fish resistance and increase their suitability for infection [24]. The outbreaks were mainly associated with a temperature higher than 15 °C, pH more than 8, oxygen saturation less than 70% [25]. The chemotactic effect exerted on *V. alginolyticus* and their ability to adhere to external body surface was significantly increased at a higher temperature, which thereby enhanced the entry of that pathogen into the host [10]. Similarly, [8] declared that Vibrios outbreak frequently occurred when the temperature reading above 15 °C.

Concerning the prevalence of *V. alginolyticus* in the different internal organs of infected species, our results were in agreement with those obtained by [15, 16] who declared that *Vibrio* was the most prominent species isolated mainly from the liver, kidney, and spleen of diseased fish. These findings affirmed that both liver and kidney are the principal target

organs of disease. From the pathophysiological point of view, this tissue inclination may be related to certain virulence tool owned by bacteria, which enhance their septicemic existence into the detoxification organ (liver) and a main immune worrier (kidney).

Utilizing traditional techniques as an instrument for pathogen recognition may fall shorten when a bacterium show uncommon phenotypic profiles, besides they require long time to peruse. Based on these arguments, molecular-based techniques were recently used to recognize most of the threatening pathogens in aquaculture [11]. Concerning the genetic typing of the isolated *V. alginolyticus* strains, PCR was used for detection of 16S rRNA gene which is conserved in *V. alginolyticus,* in order to confirm their diagnosis, in agreement with this obtained by [2].

Concerning the antibiotic susceptibility, the current study revealed that all examined strains showed different patterns of sensitivity to ciprofloxacin, florfenicol, and nalidixic acid, while they were resistant to erythromycin and sulpha-trimethoprim. These results were nearly agree with those obtained by [38]. The resistance of *V. alginolyticus* to erythromycin and sulfa may be attributed to the presence of *ermB* and *sul2* genes among evaluated strains, which still required further investigation. Alternatively, it may due to the rhythmic existence of such antibiotics in waters of the sampling localities because of agricultural/municipal drainage, which may contain antibiotic wastes of the same types [1].

Regarding the challenge trial, all infected fish species showed nearly the same clinical signs and postmortem lesions found in naturally infected one. Most of experimentally infected fish died within 5-10 days post inoculation. The rapid onset of *T. zillii* death, was attributed to the lethal and toxic effect of bacterial toxins and their extracellular products, which in turn induce tissue and cell damage [21]. On the other side, the mortality rate in *O. niloticus* was relatively low (70%) in compare to *T. zillii*, and extended for up to 10 days. These results reflected the role of water salinity on the disease occurrence, which may be clarified by the typical inhabitants of *Vibrio* spp in seawater, sediment, and also in the alimentary tract of marine fish [33]. The results were in accordance with those obtained by [5] who reported that mortality rate of experimentally infected fish with *V. alginolyticus* was 50% within 10 days after injection.

In conclusion, *V. alginolyticus* is highly pathogenic to freshwater and saltwater fish and considered a critical sanitary problem to their culture. Combination of both phenotypic and genotypic characterization is a reliable and vulnerable tool for diagnosis of *V. alginolyticus*. The massive increase in the antibiotic resistance phenomena among various threatening pathogens recently becomes a critical point of public health concern, so monitoring the antibiotic sensitivity of such isolate was vital to select the most effective antibiotic.

Conflict of interest

The authors pronounce that they have no huge contending budgetary, expert or individual interests that may have affected the execution or introduction of the work.

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