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Vibrio pathogenicity island and phage *CTX* genes in *Vibrio alginolyticus* isolated from different aquatic environments

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ABSTRACT

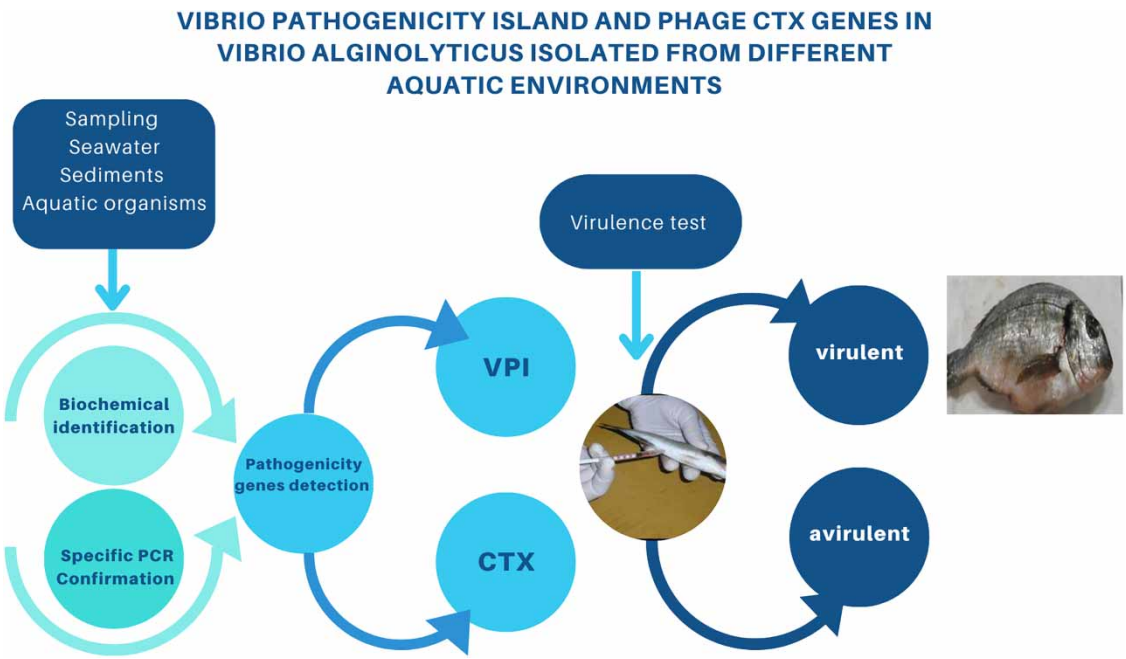
In the present study, we investigated the presence of four *Vibrio cholerae* virulence genes (*ctxA*, *VPI*, *Zot* and *ace*) in 36 *Vibrio alginolyticus* isolates obtained from different seawater, sediments and aquatic organisms. We tested the virulence of 13 *V. alginolyticus* strains against juveniles of *Sparus aurata* and this virulence was correlated with the presence of *V. cholerae* virulence genes. A positive amplification for the virulence pathogenicity island was produced by five *V. alginolyticus* strains and four for cholerae toxin. Some of the *V. alginolyticus* strains are pathogenic to aquatic animals and might have derived their virulence genes from *V. cholerae*. *V. alginolyticus* strains can be considered as a possible reservoir of *V. cholerae* virulence genes.

Key words: *ctxA*, diseases, *Vibrio alginolyticus*, *Vibrio cholerae*, VPI

HIGHLIGHTS

- We investigated the presence of virulence genes in *Vibrio alginolyticus* strains.
- We detected the presence of the virulence markers VPI and *ctxA*.
- Strains showing VPI genes were found virulent for *Sparus aurata*.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Bacteria of the genus *Vibrio* are indigenous to the marine environment and temporarily in abundance in the ocean, being able to cause infections in humans and commercially important species of crustaceans, bivalves and fish. Severe outbreaks of diseases caused by *Vibrio* have large economic consequences for aquaculture and other economic sectors (Liu *et al.* 2004). *Vibrio alginoliticus* is a marine bacterium, and some strains can cause gastroenteritis in humans through the consumption of contaminated seafood. The transmission of *V. alginolyticus* strains from the aquatic environment, fishes and shellfish to humans has been well documented (Xie *et al.* 2005; Zhang & Austin 2005; Khouadja *et al.* 2012). This bacterium is frequently associated with mass mortality of *Sparus aurata* and *Dicentrarchus labrax* larvae and older fish in many Tunisian hatcheries installed along the Mediterranean seacoasts (Ben Kahla-Nakbi *et al.* 2009; Snoussi *et al.* 2009), and this pathogen was also reported in many other countries in Mediterranean coasts (Zanetti *et al.* 2000; Croci *et al.* 2001; Balcazar *et al.* 2009).

ctxAB, encoding for cholerae toxin (CT) and the *V. cholerae* pathogenicity island (*VPI*) is associated with the epidemic strains in *V. cholerae* (Waldor & Mekalanos 1996). The *ctxAB* genes are carried in the genome of a filamentous, single-stranded DNA phage-designated CTX, and their dissemination to non-pathogenic strains may, therefore, occur via phage-mediated horizontal gene transfer (Waldor & Mekalanos 1996). The *VPI* has recently been proposed to also be a filamentous phage VPIØ (Li *et al.* 2003). Unlike for the CTXØ, convincing data are lacking for the existence or the horizontal transfer of VPIØ. At present, none of the 29 genes in the *VPI* (other than *tcpA*) has been assigned, based on experimental data, any function in the proposed phage's life cycle or phage transduction (Faruque *et al.* 2003; Krebs & Taylor 2011). As it has been suggested that *Vibrio* strains may arise from toxigenic *V. cholerae TCP* and *ToxR* by infection with CTXØ and virulence genes reported in other *Vibrio* strains, a need was identified for an investigation into the dissemination of these genes among other *Vibrio* species.

In the present study, we investigated the presence of four *Vibrio cholerae* virulence genes (*ctxA*, *VPI*, *Zot* and *ace*) in 36 *Vibrio alginolyticus* isolates obtained from different seawater, sediments and varied aquatic organisms. In addition, we tested the virulence of 13 *V. alginolyticus* strains to see if there is a correlation between the presence of certain virulence genes and the pathogenicity.

2. MATERIALS AND METHODS

The samples were taken from different sites along the Tunisian seacoasts (Table 1) and were transported on ice to the laboratory within 2 h of sampling and processed for bacterial identification.

Samples were analyzed according to a standard procedure (Ottaviani *et al.* 2003). Briefly, 25 g of blended sample was inoculated in 225 ml of alkaline peptone-water (Difco, Spain) with 3% NaCl and incubated at 30 °C for 18–24 h. Liquid samples were filtered (0.45 μm, Millipore, Sartorius Minisart CE 0297, Germany) and membranes were enriched on alkaline peptone-water (3% NaCl) and then incubated at 30 °C for 18–24 h. A 10 μl of the enrichment culture was streaked onto Thiosulphate-citrate-bile salt-sucrose-modified agar (TCBS agar) used for the selective isolation of *Vibrio* strains (Scharlau Microbiology, Spain). After 18–24 h of incubation at 30 °C, only the cultures giving pure yellow colonies (2–3 colonies from each sample) were randomly selected and then subcultured on Tryptic soy agar (TSA, Difco, Spain) supplemented with 3% NaCl (Hara-Kudo *et al.* 2001). The isolated bacteria were frozen at −80 °C with 20% (v/v) glycerol for further analysis.

Table 1 | Number of samples, origin and source

Source	Species	No. of sample	No. of strains	Origin
Shellfish	*Crassostrea gigas*	10	2	The Lake of Bizerte northern Tunisia
	Mytilus edulis	10	1	The Lake of Bizerte northern Tunisia
Fishery products	*Dicentrarchus labrax*	10	6	Sousse, Centre of Tunisia
	Sparus aurata	10	8	Sousse, Centre of Tunisia
	Solea solea	10	2	Sousse, Centre of Tunisia
	Liza aurata	10	4	Sousse, Centre of Tunisia
Environmental	Seawater	60	6	The different sites sampled
	Sediment	60	7	The different sites sampled
Human			1	(ATCC 17749) Japan

2.1. Biochemical characterization

Yellow colonies on the TCBS (2–3 colonies from each sample) were randomly selected and subcultured on TSA supplemented with 3% NaCl (TSA, Difco, Spain) and standard procedures were used for the determination of Gram, cell morphology, the oxidase, catalase, indole production, O–F test, motility (Mannitol-Motility agar, Pronadisa, Madrid, Spain), susceptibility to the vibriostatic compound O/129 (10 and 150 μg/disc), were the first tests employed to identify the organisms belonging to *Vibrio* genus. The API 20NE (bioMerieux) procedure was modified in order to incorporate a 2.5% NaCl concentration in all microtubes. An initial bacterial suspension was therefore prepared in 5 ml of a 2.5% NaCl solution instead of the recommended 0.85% NaCl medium. Incubation time and temperature were maintained within the limits prescribed (37 °C for 24 h) (Croci *et al.* 2007). Identification was obtained through the APILAB PLUS software (bioMerieux) and was considered acceptable when giving a probability equal to or greater than 80% (Croci *et al.* 2007).

2.2. Confirmation by specific PCR

Bacteria cultured on 3% NaCl tryptone soya broth (TSB-S, Oxoid) at 30 ± 1 °C for 24 h were extracted by boiling. *V. alginolyticus* was tested for collagenase gene according to Di Pinto *et al.* (2005). Primer sequences and region amplified are summarized in Table 2. All reactions were performed in a volume of 25 μl on a 9600 Applied Biosystems thermalcycler. *V. alginolyticus* ATCC 17749 was used as the positive control for collagenase gene detection; a negative control (sterile distilled water) was processed for every 10 strains. Polymerase chain reaction (PCR) products were visualized by electrophoresis on 1.5% agarose gel (Kodak, New Haven, CT, USA) (run at 90 V for 50 min) and photographed using a Bio-Rad Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA).

In order to exclude the misidentification of other species like *V. alginolyticus*, the results of all screening and biochemical tests were compared with those available in the literature for the species with closest phylogenetic relations according to previously published papers (Thompson *et al.* 2005). Misidentification with certain species such as *V. cholerae* (Nandi *et al.* 2000), *V. vulnificus* (Brauns *et al.* 1991), *V. parahaemolyticus* (Kim *et al.* 1999) and *V. harveyi* (Dalmasso *et al.* 2009) was also excluded by subjecting the isolates to the PCR assays for the identification of these species.

2.3. Protease and haemolytic activities

Protease activities were analysed by spot inoculations on TSA 3% NaCl supplemented with skimmed milk (2% wt/vol), (Alcaide *et al.* 1999; Hormansdorfer *et al.* 2000). Haemolysin potency was evaluated using a modification technique of

Table 2 | List of selected oligonucleotide primers

	Target	Primers	Amplicon size	References
V. parahaemolyticus	*toxR*	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	366 bp	Kim *et al.* (1999)
V. alginolyticus	*collagenase*	CGAGTACAGTCACTTGAAAGCC CACAACAGAACTCGCGTTACC	737 bp	Di Pinto *et al.* (2005)
V. cholerae	*ompW*	CACCAAGAAGGTGACTTTATTGG GAACTTATAACCACCCGCG	587 bp	Nandi *et al.* (2000)
V. harveyi	*16S rRNA*	AACGAGTTATCTGAACCTTC GCAGCTATTAACTACACTACC	180 bp	Dalmasso *et al.* (2009)
V. vulnificus	*vvh*	CGCCGCTCACTGGGGCAGTGGCG CCAGCCGTTAACCGAACCACCCC	387 bp	Brauns *et al.* (1991)
Zot	*Zot*	CGTCTCAGCATCAGTATCGAGTT ATTTGGTCGCAGAGGATAGGCCT	198 bp	Colombo *et al.* (1994)
ctxA	*ctxA*	CGGGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCCAC	563 bp	Fields *et al.* (1992)
ace	*ace*	GCTTATGATGGACACCCTTTA TTTGCCCTGCGAGCGTTAAAC	289 bp	Colombo *et al.* (1994)
VPI	*VPI*	GCAATTTAGGGGCGCGACGT CCGCTCTTTCTTGATCTGGTAG	680 bp	Sechi *et al.* (2000)

the plate assay described previously (Quindos *et al.* 1994). In brief, 10 µl of suspension (10^8 cells/ml) were spotted onto human blood and fish blood agar made by mixing 70 ml of each blood with 1,000 ml (TSA) supplemented with 3% NaCl (Khouadja *et al.* 2012). The plates were incubated at 30 °C for 24 h. Positive haemolytic potency was recorded by the presence of a distinct translucent halo around the inoculum area. The diameters of the zones of lysis and the colony were measured and the ratio (equal to or larger than 1) was used as a haemolytic index to represent the intensity of haemolysin production by the tested strains. All the tests were repeated three times.

2.4. Detection of phage *CTX* genes and pathogenicity genes

All isolated identified *V. alginolyticus* strains were grown overnight at 37 °C on a TSA supplemented with 3% NaCl. All primers used in this study are summarized in Table 2. Bacterial DNA for PCR analysis was extracted with a Wizard Genomic DNA Purification kit (Promega). Amplification reactions were performed in a 25-µl reaction mixture containing 3 µl of genomic DNA, 5 µl of Green GO Taq buffer (5×), 200 mM of each deoxynucleoside triphosphates (dNTP), 25 µM of each primer and 1 U of GO Taq DNA polymerase (Promega, USA). The mixtures were incubated for 5 min at 94 °C, followed by 35 cycles of amplifications. Each cycle of amplifications consisted of a denaturation step at 94 °C for 40 s, annealing for 40 s and primer extension for 1 min at 72 °C. The mixtures were kept at 72 °C for 10 min for the final extension. The annealing temperatures were as follows: 60 °C for *zot* and *ctxA* and 62 °C for *ace* and *VPI* genes. *CTX*- and *VPI*-positive amplicons to be sequenced were directly purified from PCR tubes or extracted from agarose gel using Wizard SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions. DNA sequences were determined by Bio-fab (Rome, Italy). CLC Genomics Workbench 3 software was used to align bidirectional DNA sequences, which were subsequently blasted and analyzed against the GenBank database.

2.5. Virulence test

The median infective dose (ID_{50}) test was conducted by intraperitoneal (i.p.) injection as previously described (Alcaide *et al.* 1999). *V. alginolyticus* strains showing the presence of *CTX* gene ($n = 4$), the five strains with *VPI* genes and four strains which did not carry any of the investigated genes were grown overnight in TSA 3% NaCl at 30 °C, from each one, a colony was subcultured in 40 ml fresh medium (TSB 3% NaCl) at 30 °C for 16 h.

The cells were harvested by centrifugation (5,000 rpm, 10 min), washed and resuspended in phosphate buffer saline (PBS; 0.01 M) to OD_{600} of 0.2–0.9, so that the bacterial concentrations were 10^2–10^8 cfu ml^{-1} determined by the dilution-plate method. Sterile PBS was injected i.p. into fish as a control.

Healthy juveniles of *S. aurata* (weight 5 g, length 8 cm, 20 individuals per testing dose) from a commercial fish farm were randomly sampled for the experiment and acclimated in the water at 25 °C for 4–5 days before testing; water was kept at salinity of 37‰ and under continuous aeration. Before infection, the juveniles were fed with commercial food (INVE Aquaculture Nutrition), while feeding was suspended during the virulence test. Each bacterial dilution containing from 10^2 to 10^8 cfu ml^{-1} was tested by i.p. injection of 50 µl of suspension (20 individuals per testing dose). Sterile PBS was injected i.p. as a negative control. Mortalities were recorded daily for 7 days and were only considered infected if *V. alginolyticus* was recovered from assayed fish.

The ID_{50} was calculated by a simple method for estimating 50% endpoints (Reed & Muench 1938). The percentage of infected fish is calculated as follows: $A/(A + B) \times 100$ with A corresponding to infected fish and (B) to not infected fish. The Reed Muench formula is used to calculate the index: index = (% infected fish at dilution immediately above 50%–50%)/(% infected at dilution immediately above 50%–% infected at dilution immediately below 50%). Applying the index calculated using this formula to the dilution that produced the infection rate immediately above 50%, we obtained the dilution of inoculum producing 50% infection. The entire experiment was under ethical approval, and fish were subject to independent health checks during the work.

3. RESULTS

3.1. Biochemical and molecular identification

V. alginolyticus is present in a broad range of aquatic environments and marine organisms (Table 1). All 36 strains collected, including the reference strain, were Gram-negative, motile, pleomorphic, mostly coccobacillary rods. They grew as unpigmented, moist, swarming colonies. All the strains grew in peptone water prepared, respectively, with 3%, 8%, and 10% of NaCl.

Api 20NE strips demonstrated the heterogeneity of *V. alginolyticus* populations studied, out of 36 *V. alginolyticus* strains tested, 22 biotypes were identified. The collagenase-based PCR (Di Pinto *et al.* 2005) allowed confirmation of the phenotypic identification for the 36 strains isolated. The isolates identified as *V. alginolyticus* by conventional procedures produced an amplicon of 737 bp (Figure 1), characteristic of this species.

3.2. Detection of pathogenicity genes

The PCRs of four virulence genes (*zot, ace, VPI* and *ctxA*) were conducted for the 36 *V. alginolyticus* strains. Five strains showed a positive amplification for the pathogenicity island (*VPI*) (Figure 2) and four for *CTX* genes (*ctxA*) (Figure 3). In order to confirm the *CTX*- and *VPI*-positive amplification, we purified, sequenced and aligned the sequence of this amplicon. Based on the sequence obtained from these genes and their alignment with other gene sequences recovered from the NCBI database, we concluded that sequences are homologous to those of *V. cholerae*. The present sequences showed 94% identity with *VPI* (GenBank accession no. KC677670) and 100% identity with cholera enterotoxin subunit A (*ctxA*) (GenBank accession nos KC677671 and KC677672). All strains tested were negative for the amplification of both Zonula occludens toxin (*zot*) and *ace* genes.

3.3. Virulence tests

All haemolytic strains were virulent and caused mortality during the infection tests. Pure culture of *V. alginolyticus* was isolated from the liver and skin of moribund fish and no mortality was detected in the control group. Signs of infection such as lethargic swimming and external lesions began to appear on the second day with *VPI*-positive strains (Figure 4). S4, S8, S9, S13, S15, S16 and S20 strains did not induce any infection signs. The most pathogenic strains were the strains with *VPI* genes

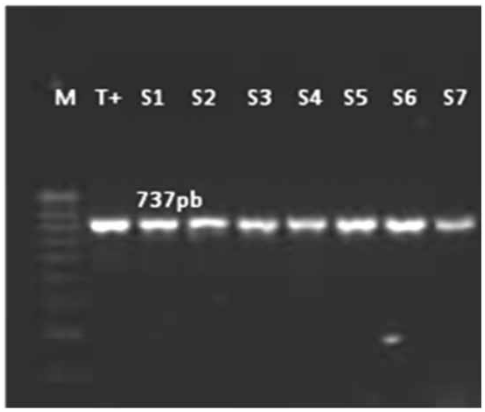


Figure 1 | Detection of collagenase gene of *Vibrio alginolyticus* by using PCR. *M*, molecular mass marker 100 bp; *T* +, controls + *V. alginolyticus ATCC 17749*; S1–S7, environmental strains.

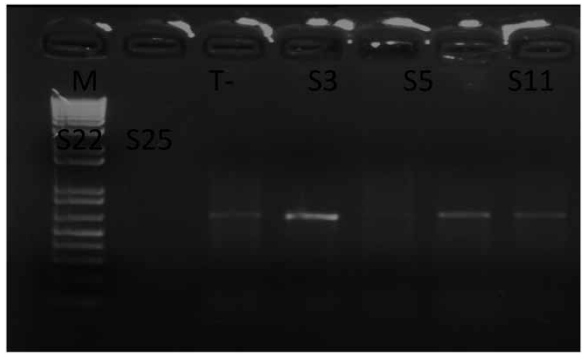


Figure 2 | Detection of *VPI* gene of *Vibrio alginolyticus* using PCR. *M*, molecular mass marker; *T*–, controls–; S3, S5, S11, S22 and S25, environmental strains.

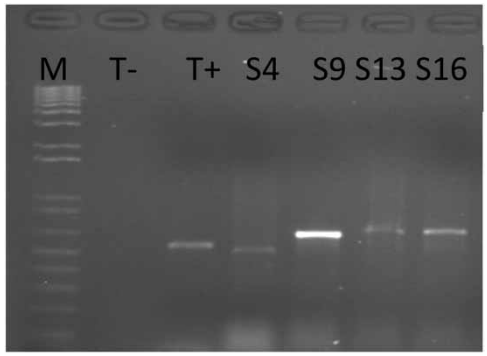


Figure 3 | Detection of *ctxA* gene of *Vibrio alginolyticus* by PCR. *M*, molecular mass marker; *T*–, controls–; *T* +, controls + *V. cholerae* O1 biovar El Tor N16961; S4, S9, S13 and S16, environmental strains.

Figure 4 | Signs of infection observed on *Sparus aurata* including skin haemorrhages and necroses after infection tests.

S11 and S25 with an ID_{50} of 1.52×10^3 and 1.96×10^3 cfu fish^{-1}, respectively (Table 3). S22 strain shows an ID_{50} of 1.89×10^3 cfu fish^{-1} but a lower haemolytic index than strains S11 and S25.

4. DISCUSSION

V. alginolyticus is present in a broad range of aquatic environments and marine organisms (Table 1). Several studies have demonstrated that these miniaturized biochemical tests, especially API 20 E and 20 NE, were used with success to identify bacteria belonging to the *Vibrionaceae* family (O'Hara *et al.* 2003; Croci *et al.* 2007). But certain environmental strains can produce atypical reactions which will give unacceptable profiles. The molecular approach allows researchers to overcome the drawbacks of traditional biochemical methods because those can be distorted by atypical reactions or by the underestimation of the number of viable cells.

Prominent virulence factors of *V. alginolyticus* are extracellular, proteolytic enzymes and haemolysines (Zhang & Austin 2005). In addition to caseinase, gelatinase and alkaline serine protease, tissue-damaging collagenase as well as amylase, lecithinase and lipase are produced. 80% of food isolates of *V. alginolyticus* produce extracellular components toxic to animal cells, fish, crustaceans and corals (Wong *et al.* 1992; Hormansdorfer *et al.* 2000; Ben Kahla-Nakbi *et al.* 2009). Certain isolates were reported to exhibit variation in one or two biochemical reactions and exhibited deviation from the standard methodology in giving few biochemical reactions (Dileep *et al.* 2003). However, due to the presence of both false-positive or false-negative results in all the biochemical identification methods proposed, some authors (O'Hara *et al.* 2003; Croci *et al.* 2007) suggested caution in the interpretation of identifications and advised additional confirmatory testing, such as PCR. Conventional culture-based

Table 3 | *Vibrio alginolyticus* strains used in the virulence test

Strains	Origin	Api 20 NE	Identification Api 20 NE (%)	Season	zot	ctxA	ace	vpi	Protease	Haemolysis		ID_{50} (cfu $fish^{-1}$)
										(H)	(S)	
S1	B	7454444	99.9%	*Winter*	−	−	−	−	−	*<1*	*>1*	ND
S2	*B*	7444444	99.9%	*Winter*	−	−	−	−	−	*<1*	*>1*	ND
S3	*B*	7136144	33.33%	*Winter*	−	−	−	+	+	*>1*	*>1*	2.33×10^5
S4	*C*	4067134	33.33%	*Winter*	−	+	−	−	−	*<1*	*<1*	avirulent
S5	*B*	4047134	Unacceptable	*Spring*	−	−	−	+	+	*>1*	*>1*	6.1×10^5
S6	*B*	3746244	66.67%	*Spring*	−	−	−	−	−	*<1*	*>1*	ND
S7	*H*	5107124	Unacceptable	*Spring*	−	−	−	−	−	*<1*	*>1*	ND
S8	*C*	7446144	Unacceptable	*Spring*	−	−	−	−	−	*>1*	*<1*	avirulent
S9	*B*	7073345	Unacceptable	*Summer*	−	+	−	−	+	*<1*	*<1*	avirulent
S10	*A*	7053345	Unacceptable	*Summer*	−	−	−	−	+	*<1*	*>1*	ND
S11	*D*	7053344	Unacceptable	*Summer*	−	−	−	+	−	*>1*	*>1*	1.52×10^3
S12	*E*	7053304	Unacceptable	*Summer*	−	−	−	−	−	*<1*	*<1*	ND
S13	*D*	7414444	99.9%	*Summer*	−	+	−	−	−	*<1*	*<1*	avirulent
S14	*C*	7346144	Unacceptable	*Summer*	−	−	−	−	−	*<1*	*<1*	ND
S15	*E*	7446434	99.9%	*Summer*	−	−	−	−	+	*<1*	*>1*	avirulent
S16	*D*	7746144	33.33%	*Summer*	−	+	−	−	−	*<1*	*<1*	avirulent
S17	*D*	7053734	33.33%	*Summer*	−	−	−	−	−	*<1*	*<1*	ND
S18	*E*	7057334	33.33%	*Summer*	−	−	−	−	−	*<1*	*>1*	ND
S19	*F*	7446414	33.33%	*Autumn*	−	−	−	−	+	*>1*	*<1*	avirulent
S20	*F*	7446144	Unacceptable	*Autumn*	−	−	−	−	+	*>1*	*<1*	avirulent
S21	*A*	7053375	83.33%	*Winter*	−	−	−	−	+	*>1*	*<1*	ND
S22	*G*	7012305	83.33%	*Winter*	−	−	−	+	+	*<1*	*<1*	1.89×10^3
S23	*G*	7012315	83.33%	*Winter*	−	−	−	−	+	*<1*	*<1*	ND
S24	*B*	7012115	83.33%	*Spring*	−	−	−	−	+	*<1*	*<1*	ND
S25	*B*	7436144	Unacceptable	*Spring*	−	−	−	+	+	*>1*	*>1*	1.96×10^3
S26	*E*	7346144	Unacceptable	*Spring*	−	−	−	−	+	*<1*	*<1*	ND
S27	*C*	7746144	Unacceptable	*Spring*	−	−	−	−	+	*<1*	*<1*	ND
S28	*C*	5053365	Unacceptable	*Spring*	−	−	−	−	+	*<1*	*<1*	ND
S29	*C*	5053365	Unacceptable	*Spring*	−	−	−	−	+	*<1*	*<1*	ND
S30	*D*	7446444	99.9%	*Spring*	−	−	−	−	+	*<1*	*<1*	ND
S31	*A*	7426144	99.9%	*Spring*	−	−	−	−	+	*<1*	*<1*	ND
S32	*A*	7436144	99.9%	*Winter*	−	−	−	−	+	*<1*	*<1*	ND
S33	*A*	7446434	99.9%	*Winter*	−	−	−	−	+	*<1*	*<1*	ND
S34	*D*	7454444	Unacceptable	*Winter*	−	−	−	−	+	*<1*	*<1*	ND
S35	*A*	7446444	Unacceptable	*Spring*	−	−	−	−	+	*<1*	*<1*	ND
S36	*D*	5053345	Unacceptable	*Autumn*	−	−	−	−	−	*<1*	*<1*	ND
ATCC 17802	*Human*	7454444	99.9%		−	−	−	−	+	*>1*	*<1*	ND

ND, not determined; H, human erythrocytes; S, *Sparus aurata* erythrocytes; >1, superior to 1 cm; <1, inferior to 1 cm; A, *Dicentrarchus labrax*; B, *Sparus aurata*; C, Seawater; D, Sediment; E, *Liza aurata*; F, *Solea solea*; G, *Crassostrea gigas*; H, *Mytilus edulis*.

methods for the detection of *V. alginolyticus* are laborious and time-consuming (>2 days). Recently, PCR-based methods were developed, which can be completed within 1 day (Zhou *et al.* 2007). The PCR assay based upon the detection of the gene coding for collagenase could be used as an alternative molecular target for *V. alginolyticus* detection.

V. cholerae is known to be responsible for the severe diarrhoeic disease that continues to be a global threat to human health. Different genes play a role in the expression of the potent CT (Fields *et al.* 1992), which is encoded by the *ctxAB* genes on the filamentous phage *CTX* along with genes encoding other virulence factors such as the Zonula occludens toxin (*Zot*), accessory cholera enterotoxin (*Ace*) and a core-encoded pilin (*cep*) (Colombo *et al.* 1994; Lipp *et al.* 2003). Different studies demonstrated that *V. parahaemolyticus* and *V. cholerae* pathogenicity-associated genes may be transferred to different *Vibrio* spp. strains isolated from the coastal waters (Sechi *et al.* 2000; Zhou *et al.* 2007; Ben Kahla-Nakbi *et al.* 2009). It is now clear that in aquatic biotopes, *V. alginolyticus* might be continually undergoing genetic change by the acquisition of DNA originating from *V. cholerae* strains.

Our results show that nontoxigenic strains do not present any of the virulence genes. Some, including *ctxA* and *tdh*, may be horizontally transferred, leading to new pathogenic strains. *V. alginolyticus* strains often possess homologues of the *V. parahaemolyticus* and *V. cholerae* virulence genes such as *toxR*, *tlh* and *VPI* (Xie *et al.* 2005), which suggests that *V. alginolyticus* may be an important reservoir of many known virulence genes of other *Vibrio* species in the aquatic environment. It is probable that the aquatic environment harbours different virulence-associated genes scattered among environmental *Vibrio*.

In the present study, the five strains with *VPI* genes showed beta haemolytic activity, when the other isolates were found to exhibit weak haemolysis and none of the strains positive for the *ctxA* genes was found to exhibit haemolytic activity. Several negative strains for the different virulence genes showed a low haemolytic activity. The weak haemolysis points towards the presence of other virulence factors. This is confirmed during the determination of the ID_{50}, when all strains showing haemolytic activity are virulent and cause mortality during the infection tests. When compared with other infectivity assays by the same species in another study, the ID_{50} in *S. aurata* was ranging from 2×10^5 to 6.2×10^5 cfu fish^{-1} (Xie *et al.* 2005; Ben Kahla-Nakbi *et al.* 2009), which is very similar to S3 and S9 strain values, whereas others strains, such as S11, S22 and S25 are still deadly with much lower ID_{50} values. In our study, strains with *VPI* genes appear to have greater infective power compared to the other strains tested.

The pathogenicity island is a large unstable chromosomal region that encodes several virulence genes, and is present in human pathogenic isolates and absent from non-pathogenic isolates (Hacker *et al.* 1997). The genes encoding the biosynthesis of *TCP* were initially shown to reside on a pathogenicity island, designated the *VPI* (Karaolis *et al.* 1998). The fact that some strains exhibit none of the sought genes is pathogenic for fish, and can be regarded as a synergistic action of *V. alginolyticus* enzymes. Prominent virulence factors correlated with the pathogenicity of many *Vibrio* species are extracellular, proteolytic enzymes and haemolysines (Zhang & Austin 2005). In addition to caseinase, gelatinase and alkaline serine protease, tissue-damaging collagenase as well as amylase, lecithinase and lipase are produced (Hormansdorfer *et al.* 2000). These factors could facilitate the propagation of the bacteria by causing extensive host tissue damage, thereby degrading host proteins that provide readily available nutrients for bacterial growth (Balebona *et al.* 1998). However, the pathogenic mechanism of *V. alginolyticus* is not completely understood. Analysis of the relationship between virulence-associated genes and pathogenicity of *V. alginolyticus* provides a possible explanation for why the pathogenic mechanism of *V. alginolyticus* might be different from that of *V. parahaemolyticus* and *V. cholerae*. This suggests that *V. alginolyticus* might have a different virulence gene system and different pathogenic mechanism compared with *V. cholerae*, despite adhesion and hydrolytic activities. The difference in the hydrolytic enzymes produced by these strains can affect the virulence and they differ one from the other. This can explain the difference among inter- and intra-specific mechanisms of virulence, suggesting the specificity of virulence can be strain-specific.

Different studies demonstrate that the death process induced by *V. alginolyticus* in mammalian cells is different from that in fish cells, including induction of autophagy, cell rounding and osmotic lysis (Zhao *et al.* 2011; Ren *et al.* 2013). *V. alginolyticus* requires its type III secretion system (T3SS) to cause rapid death of infected fish cells and infection with this species also led to membrane pore formation and release of cellular contents from infected fish cells, as evidenced by lactate dehydrogenase release and the uptake of a membrane-impermeable dye (Zhao *et al.* 2010, 2011).

5. CONCLUSIONS

In our study, 5 of 13 strains tested for virulence properties were able to cause mortality in juveniles of *S. aurata*. This strongly supports the fact that individual strains of *V. alginolyticus* are pathogenic to aquatic animals. However, our results do not show an obvious correlation between the presence of *ctxA* genes and virulence, whereas in all *VPI*-positive strains, the virulence was established.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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