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RT-PCR assays for in vivo expression of *Vibrio alginolyticus* virulence genes in cultured gilthead *Dicentrarchus labrax* and *Sparus aurata*

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Abstract - In this study, we investigated the pathogenic potency of two *Vibrio alginolyticus* strains isolated from cultured gilthead *Dicentrarchus labrax* and *Sparus aurata*, in Tunisia. The enzymatic activities of the extracellular products of the studied strains characterized with the API-ZYM system, revealed the existence of several activities. The 50% lethal doses levels were then determined, virulence levels ranged from 1.03×10^5 to 1.0×10^6 CFU/fish. In addition, we also searched for the presence of eight *Vibrio cholerae* virulence genes: *ctxA*, *zot*, *ace*, *toxR*, *toxS, toxRS, toxT*, and Virulence Pathogenicity Island (VPI), in the genome of two isolated strains of *V. alginolitycus*. The *in vivo* expression of *toxR, toxS,* VPI and *ace* genes in both fish species, was tested by reverse transcriptase polymerase chain reaction. Indeed, we have noted that for each strain all the virulence genes selected were expressed in sea bream as well as in sea bass.

Key words: *Vibrio alginolyticus*; *Dicentrarchus labrax*; *Sparus aurata*; LD_{50}; virulence genes expression; RT-PCR.

INTRODUCTION

Sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata.* L) are marine fish with high economic value in the Mediterranean aquaculture farms. Several pathogenic microorganisms have been isolated from outbreaks affecting these fish species (Ben Kahla *et al*., 2006). One of these organisms, *Vibrio alginolyticus*, is frequently involved in epizootic outbreaks in cultured gilt-head sea bream and sea bass, causing fish mortality and important economic losses (Zorrilla *et al*., 2003). Besides its pathogenicity for Human, this bacterium belongs to the most important pathogens in aquaculture, causing tremendous damage in shellfish and crustaceans (Hörmansdorfer *et al*., 2000). In addition, this *Vibrio* species is considered as the causal agent of outbreaks of vibriosis in *Epinephelus malabaricus* (Lee, 1995) and sea bream (Faruque and Nair, 2002) and also has been associated with other *Vibrio* species in high-mortality outbreaks related to abdominal swelling in larvae of several fish species (Sedano *et al*., 1996).

Several studies investigated the dissemination of some *V. cholerae* and *V. parahaemolyticus* virulence genes among *V. alginolyticus* strains (Sechi *et al*., 2000, Xie *et al*., 2005). According to Boyd *et al*. (2000) these genes may be horizontally transferred to *V. alginolyticus* in an aquatic environment. Indeed, the mobility of virulence genes may cause the transformation of non pathogenic strain to pathogenic strain. Xie *et al*. (2005) reported that *V. alginolyticus* often possess homologues of the *V. parahaemolyticus* and *V. cholerae* virulence genes such as *toxR*, *tlh* and VPI, suggesting that *V. alginolyticus* may be an important reservoir of many known virulence genes of other *Vibrio* species in the aquatic environment. It is probably that the aquatic environment harbours different virulence-associated genes scattered among environmental Vibrios.

The aim of this work was to evaluate the virulence potential of two *V. alginolyticus* strains responsible for outbreaks that lead to mortality in cultured gilt-head sea bream and sea bass in Tunisian aquaculture farms. The extracellular products were characterized with the API-ZYM system. Others exoenzymes have been also investigated. In order to study the in vivo expression of virulence genes reverse transcriptase polymerase chain reaction (RT-PCR) method was used.

MATERIALS AND METHODS

Bacterial strains isolation. Two *V. alginolyticus* strains (S1 and S2) were isolated from diseased gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), respectively, according to the method described previously (Ben Kahla *et al*., 2006). Fish species had a weight around 10 g and were collected

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TABLE 1 - PCR primers selected for this study (Sechi *et al*., 2000)

Oligonucleotide sequence	Amplification region (bp)
*tox*RS toxR0, 5′-ATGAGTCATATTGGTACTTAAATT-3′ toxS2, 5′-AACAGTACCGTAGAACCGTGA-3′	1397
*tox*S toxS1, 5′- CCACTGGCGGACAAAATAACC-3′ toxS2, 5′-AACAGTACCGTAGAACCGTGA-3′	640
*tox*R toxR1, 5′-TTTGTTTGGCGTGAGCAAGGTTTT-3′ toxR2, 5′-GGTTATTTTGTCCGCCAGTGG-3′	595
VPI VPI1, 5′-GCAATTTAGGGGCGCGACGT-3′ VPI2, 5′-CCGCTCTTTCTTGATCTGGTAG-3′	680
*tox*T toxT1, 5′-TTGCTTGGTTAGTTATGAGAT-3′ toxT2, 5′-TTGCAAACCCAGACTGATAT-3′	581
ace ace1, 5′-GCTTATGATGGACACCCTTTA-3′ ace2, 5′-TTTGCCCTGCGAGCGTTAAAC-3′	284
zot zot1, 5′-ACGTCTCAGACATCAGTATCGAGTT-3′ zot2, 5′-ATTTGGTCGCAGAGGATAGGCCT-3′	198
*ctx*A ctx2, 5′-CGGGCAGATTCTAGACCTCCTG-3′ ctx3, 5′-CGATGATCTTGGAGCATTCCCAC-3′	563

from tanks of 35‰ salinity seawater and at a temperature ranging from 16 to 22 °C. Samples from liver, spleen, kidney and external lesions of diseased fish were cultured on 1% Tryptic Soy broth and 1% Tryptic Soy agar (TSB and TSA, Pronadisa, Spain) supplemented with 1% (w/v) NaCl and on Thiosulphate-Citrate-Bile salt-Sucrose agar (TCBS, Difco). All the inoculated media were incubated at 22 °C for 24-72 h. All media that showed growth in pure culture were selected for strain identification. The isolated bacteria from diseased sea bream and sea bass were identified using the Api 20E system (bio-Merieux). They were Gram-negative, oxidase positive, catalase positive, motility positive, fermentative, swarmed on 1% TSA, and producing yellow colonies on TCBS agar. The isolated bacteria were frozen at -80 °C with 20% (v/v) glycerol for further analysis.

Extraction and characterization of extracellular products. Bacterial extracellular products were obtained according to the technique described by Liu (1957). Briefly, tubes containing 5 ml of 1% TSB were inoculated with one colony from a 24 h culture on 1% TSA and incubated at 22 °C. A 200 µl portion of the culture was spread into a sterile cellophane sheet overlaying 1% TSA and incubated at 22 °C for 48 h. Bacterial cells were harvested with a Phosphate Buffered Saline (PBS, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl, at pH 7), cell suspensions were centrifuged at 10000 *x g*, 4 °C for 20 min. The supernatants were filtered through 0.45 µm and 0.2 µm-pore-size membrane filters and used as the crude extracellular product preparations. A number of enzymatic activities of the extracellular products were evaluated with the API-ZYM system (Bio-Merieux).

Amylase, lecithinase (phospholipase), caseinase and lipase were detected on 1% TSA added respectively, with: 1% (w/v) starch, 5% (v/v) egg yolk, 5% (v/v) skim milk and 1% (v/v) Tween 80 (Merck).

Virulence for fish. The 50% lethal doses (LD_{50}) for the two *V. alginolyticus* strains (S1 and S2) were determined with healthy sea bream and sea bass weighing around 10 g. LD_{50} of bacterial cells were determined after intraperitoneal inoculation (0.1 ml) of groups of eight fish with bacterial doses ranging from 10^3 to 10^8 CFU/ml. In all cases, overnight cultures of the bacterial strains to be tested, were washed by centrifugation and suspended in PBS (pH 7). Groups of control fish were inoculated with 0.1 ml of sterile PBS (pH 7). The fishes were kept in aquaria (35‰ salinity) at 22 °C for 7 days and observed for pathological signs. The LD_{50} was calculated by the highest numbers method of Reed and Muench (1938). Bacteriological analyses of dead fish were carried out in all the cases, death was considered to be caused by inoculated bacteria only if the strain used for inoculation was isolated in pure culture. Surviving fish were killed and cultured to determine whether they were possible carriers. In both cases, samples were taken from liver and kidney, and cultured in 1% TSB and 1% TSA. After incubation at 22 °C for 24-72 h, the isolated colonies, were checked for purity and identified using conventional techniques.

PCR detection of *Vibrio cholerae* virulence genes in *Vibrio alginolyticus* strains. Bacteria were cultured on 1% TSA at 28 °C for 24 h. One colony was cultured in 1% TSB during 24 h at 28 °C and 1.5 ml was centrifuged. The DNA was extracted using a Wizard Genomic purification Kit (Promega, USA) according to the manufacturer's instructions. The primers of *V. cholerae* virulence genes, used in this study, are listed in Table 1.

PCR were performed in 25 µL containing: 50 ng of extracted DNA, 5 µl green Go *Taq* buffer (5X), 0.25 of each deoxynucleoside triphosphates (10 mM), 0.5 µl $MgCl_2$ (50 mM), 1 µl of each primer (25 pM) and 1 U of GO *Taq* DNA polymerase (Promega). Reaction mixtures were incubated for 5 min at 94 °C, followed by 35 cycles at 94 °C for 45 s, annealing at 52 °C for 45 s for *toxS*, *toxR* and VPI, 72 °C for 1 min and a final extension at 72 °C for 10 min. The annealing temperature for the detection of the *toxRS* and *toxT* genes was 58 °C whereas for *ctxA*, *ace* and *zot* the temperature was 60 °C. PCR products (5 µl) were analysed on 1% agarose gels stained with ethidium bromide (0.5 mg/ml) at 90 V for 1 h and visualized under ultraviolet transillumination. All PCR positive strains, indicated the presence of the virulence genes, were confirmed by repeating the PCR three times independently.

TABLE 2 - LD_{50} and PCR detection of *Vibrio cholerae* genes in *Vibrio alginolyticus*

Strain/organ isolation	LD_{50} (CFU/fish)		PCR detection of *V. cholerae* genes in *V. alginolyticus*							
	Dicentrarchus labrax	*Sparus aurata*	*tox*RS	*tox*S	*tox*R	VPI	*tox*T	*ace*	*zot*	*ctx*A
S1 (Spleen of *S. aurata*)	6.20 x 10^5	1.03 x 10^5	-	+	+	+	-	-	-	-
S2 (Liver of *D. labrax*)	5.50 x 10^5	1.0 x 10^6	-	-	-	-	-	+	-	-

***In vivo* expression of virulence genes.** In order to study the expression of *V. alginolyticus* virulence genes in cultured sea bass and sea bream, reverse transcriptase PCR (RT-PCR) method was used. Briefly, overnight cultures of the bacterial strains were washed by centrifugation and suspended in PBS (pH 7). Gilthead sea bream and sea bass were injected with a 0.1 ml of each culture (10^6 CFU/fish). Experimental infections were carried out in aquaria with 35‰ salinity and temperature of 22 °C. Liver, spleen and kidney from moribund fish were extracted and transferred separately in 1% TSB, in order to release the attached bacteria. Total RNA was extracted immediately from 1% TSB containing fish organs (liver, spleen and kidney), by SV total RNA isolation system (Promega) according to the manufacture's instructions. The purity of cultures containing each body was tested by plating on 1% TSA for 24 h. The colonies that showed a growth were confirmed by conventional techniques.

RT-PCR was performed, in triplicate independently, using SuperScript™ One-Step RT-PCR with platinum® *Taq* kit according to the manufacturer recommendations (Invitrogen). For cDNA synthesis, 1 ng of *V. alginolyticus* RNA served as template. RT-PCR (25 µl reaction volume) was performed as follows: 50 °C for 30 min, 94 °C for 5 min, and 35 cycles at 94 °C for 45 s, annealing at 52 °C for 45 s for *toxS*, *toxR* and VPI, 72 °C for 1 min and a final extension at 72 °C for 10 min. The annealing temperature for *ace* gene was 60 °C. RT-PCR products (5 µl) were analysed on 1.5% agarose gel stained with ethidium bromide (0.5 mg/ml) at 90 V for 1 h and visualized under ultraviolet transillumination. The amplification products were photographed and their sizes were determined with 100 bp molecular size marker (Hyper Ladder II, Bioline, France).

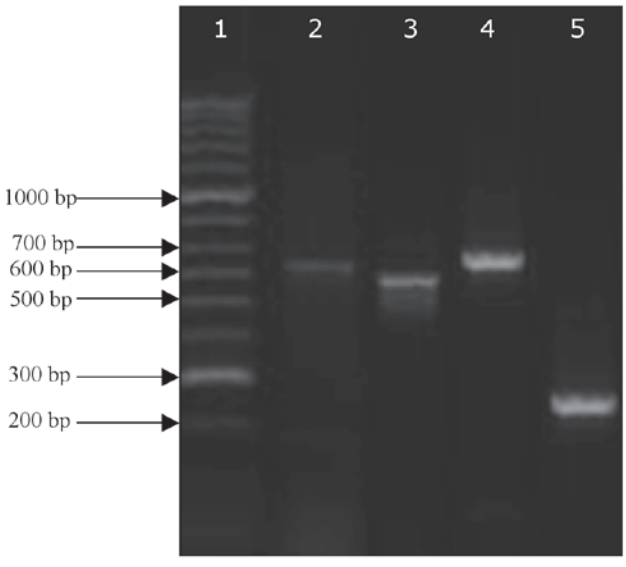

FIG. 1 - RT-PCR products from virulence genes of two *Vibrio alginolyticus* strains studied. Lane 1: 100 bp (Hyper Ladder II, Bioline), lanes 2, 3, 4 and 5 respectively *toxS*, *toxR*, VPI and *ace* genes.

RESULTS

Characterization of extracellular products

The enzymatic activities of the extracellular products characterized with the API-ZYM system revealed the same pattern for several activities. Thus, both *V. alginolyticus* strains were positive for alkaline and acid phosphatase, esterase (C4), esterase-lipase (C8), leucine arylamidase and trypsin but negative for lipase (C14), valine arylamidase, cystine, arylamidase, α-chemotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. On the other hand, expression of other exoenzymes showed that S1 as well as S2 strain was able to produce, caseinase, lecithinase, amylase and lipase.

Virulence of *Vibrio alginolyticus* strains for fish

LD_{50} assays demonstrated that all the tested strains were pathogenic for gilt head sea bream and sea bass (Table 2). LD_{50} ranged from 1.03 x 10^5 to 1.0 x 10^6 CFU/fish. Experimentally, infected fish showed external signs similar to those observed in outbreaks, including hemorrhagic fins and ulcers. Mortalities started from the first to the seventh day post-challenge, and no mortalities were observed in the group of control where fishes were injected with sterile PBS. Pure cultures of the inoculated strains were re-isolated from liver, spleen and kidney of moribund fish.

In vivo expression of selected virulence genes

The results of PCR amplification of the eight *V. cholerae* virulence genes in two *V. alginolyticus* strains showed that the strain S1 was positive for *toxR, toxS* and VPI genes. In addition, S2 was positive for the *ace* gene (Table 2). Gene expression, studied by RT-PCR, showed that all the detected genes were expressed (Fig. 1). Indeed, *toxR, toxS* and VPI genes were expressed in moribund *Dicentrarchus labrax* as well as in *Sparus aurata* for strain S1, whereas for strain S2 we have noted that accessory cholera enterotoxin (*ace*) was expressed.

DISCUSSION

The results developed in the present work showed that *V. alginolyticus* is a potential agent responsible for outbreaks that lead to mortality in cultured gilt-head sea bream and sea bass.

Extracellular products of *V. alginolyticus*, isolated from diseased fishes, contain a potent toxin such as phospholipase that probably plays an important role in the virulence mechanism of the pathogen for both poikilothermic and homoeothermic

animals (Lee *et al*., 1996). According to Maeda and Yamamoto (1996), these extracellular products, mostly consisting of proteases, could facilitate the propagation of the bacteria by causing extensive host tissue damage, thereby degrading host proteins to provide readily-available nutriments for bacterial growth. Furthermore, extracellular products could also counteract the host defense system by degrading immunoglobulins and components of the complement system.

Epizootic outbreaks, caused by *V. alginolyticus*, have been reported in gilthead sea bream (Paperna, 1984), in sea bass (Ben Kahla *et al*., 2006) and in juvenile turbot (Austin *et al*., 1993). In this study, intraperitoneal inoculation of gilt-head sea bream and sea bass with different doses of *V. alginolyticus* strains yielded LD_{50} ranging from 1.03 x 10^5 to 1.0 x 10^6 CFU/fish. Thus, the strains used can be considered highly virulent for gilt-head sea bream and sea bass on the basis of the criteria previously established (Santos *et al*., 1988). Our results are in accordance with those reported by Ben Kahla *et al*. (2006) who demonstrated that *V. alginolyticus* was pathogenic for gilthead sea bream and sea bass. Studies, carried out with several strains of *Vibrio damsela* revealed that LD_{50} ranging from 3 x 10^4 to more than 10^8 CFU/g of fish (Fouz *et al*., 1993). Balebona *et al*. (1998) reported that the LD_{50} of *V. alginolyticus* CAN for sea bream by the bath immersion inoculation technique varied depending on fish skin integrity; the LD_{50} were more than 2 x 10^7 CFU/ml for fish with intact surface layers and less than 2 x 10^3 CFU/g for fish with the mucus layer removed or the skin damaged.

Previous studies have shown the distribution of different virulence genes among *V. cholerae* O1 (Colombo *et al*., 1994, Sechi *et al*., 2000). Most *V. cholerae* strains isolated from cholera patients simultaneously carry *ctxA*, *tcp* and *toxR* genes (Faruque *et al*., 1998). Three *V. cholerae* non-O1/non-139 isolates have been found to contain the three associated virulence genes (*ctxA*, *tcpA* and *toxR*) (Ghosh *et al*., 1997). It is now clear that *V. cholerae* might be continually undergoing genetic change by the acquisition of DNA, facilitated, at least in part, by temperate phages such as those associated with some of its critical virulence factors (Sechi *et al*., 2000). Our results indicate wide dissemination among environmental *V. alginolyticus* of different *V. cholerae* virulence genes such as *toxR*, *toxS,* VPI and *ace*, 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 Vibrios. Similar results have been reported in some *Vibrio* species (Nishibuchi *et al*., 1996, Sechi *et al*., 2000). According to Boyd *et al*. (2000), these genes may be horizontally transferred, leading to new pathogenic strains. Indeed, the mobility of the virulence genes and a successful transfer may cause the transformation of a nonpathogenic strain to pathogenic strain (Faruque *et al*., 1998; Boyd *et al*., 2000). Further studies are needed to determine the nucleotide sequences of these products in order to evaluate similarity among the genes obtained from *V. alginolyticus* and *V. cholerae*, and to study their role in pathogenesis.

The present study showed the expression of *V. cholerae* virulence genes transferred to *V. alginolyticus* in moribund sea bream and sea bass. Indeed the successful transfer of virulence genes and their expression may explain the pathogenicity of the studied *V. alginolyticus* strains (S1 and S2) for cultured gilt-head sea bream and sea bass. The results of virulence genes distribution showed that there is no correlation between a virulent strain and its virulence gene genotype in *V. alginolyticus*. Indeed, although the strain S1 contain three virulence genes (*toxR, toxS and* VPI), it is less virulent than the strain S2 for sea bream who contain only *ace* gene. Similar results have been reported by Xie *et al*. (2005).

Several works have characterized the virulence factors of *V. alginolyticus* such as the extracellular product which play a very important role in the pathogenicity of this strain. This work confirmed and identified other factors of *Vibrio* pathogenicity for cultured gilt-head sea bream and sea bass. The presence and the expression of *V. cholerae* virulence genes in *V. alginolyticus* may explain the important fish mortality rate in Tunisian and in the Mediterranean aquaculture farms. Once, the informations required about the virulence factors of *V. alginolyticus*, were established, the development of an adequate vaccine, antimicrobial compounds or probiotic strains seems to be quite interesting, in order to prevent further fish infections and to solve this aquaculture problem.

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