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Molecular characterization and phylogenetic analysis of highly pathogenic *Vibrio alginolyticus* strains isolated during mortality outbreaks in cultured *Ruditapes decussatus* juvenile

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ABSTRACT

In the summer of 2008 and 2009, a series of mortalities in growing out seeds of *R. decussatus* juveniles were occurred in the eastern Tunisian littoral. Nine predominant bacterial strains were isolated from dead and moribund juveniles and characterized as *Vibrio alginolyticus*. These isolates were subjected to biochemical and molecular characterization. All the *Vibrio* strains were tested for their susceptibility against the most widely used antibiotic in aquaculture as well as, the assessment of the presence of erythromycin (*emrB*) and tetracycline (*tetS*) resistance genes among the tested bacteria. The degree of genetic relatedness between *V. alginolyticus* strains was evaluated on the basis of the Entero-Bacterial Repetitive Intergenic Consensus (ERIC) and the Random Amplification of Polymorphic DNA-PCR (RAPD-PCR) approaches. We also looked for siderophore activity and the ability to grow under iron limitation. Furthermore, the pathogenic potential of the tested isolates was evaluated using *R. decussatus* larva and juveniles as infection models. On antimicrobial susceptibility test, *Vibrio* strains exhibited total resistance to at least four antibiotics. The MICs data revealed that flumequine and oxolinic acid were the most effective antibiotics to control the studied bacteria. Results also showed that studied antibiotics resistance genes were widely disseminated in the genome of *V. alginolyticus* strains. Both ERIC and RAPD-PCR fingerprinting showed the presence of genetic variation among *Vibrio* isolates. However, RAPD typing exhibited a higher discriminative potential than ERIC-PCR. Besides, we reported here for the first time the co-production of catechol and hydroxamte by *V. alginolyticus* species. The challenge experiment showed that most of *Vibrio* isolates caused high mortality rates for both larva and juveniles at 48-h post-exposure to a bacterial concentration of 10^6 CFU/ml.

1. Introduction

Vibrio alginolyticus, is an ubiquitous bacterium in the estuaries and coastal marine ecosystems worldwide [1,2]. This species is recognized as an opportunistic pathogen causing Vibriosis outbreaks in cultured fish [3], crustaceans [4] and shellfish [5]. In Tunisia, *V. alginolyticus* was isolated for the first time in 1987 from diseased farmed gilthead sea bream and sea bass [6]. Since then, the incidence of epizootics outbreaks has been on the increase [7–9]. More recently, several cases of *V. alginolyticus* associated mortality have been reported in reared *R. decussatus* larva and juvenile causing major economic losses to the infected hatchery [10].

Highly virulent and increasingly antimicrobial resistant pathogens, such as *Vibrio* species, has become an emerging threat of increasing magnitude to the well-being of farmed aquatic animals as well as their environment. *V. alginolyticus* is generally susceptible to a wide range of antibiotics [11,12]. However, during the past decade numerous cases of multiple drug resistant strains were reported [13]. It is well documented that high frequency of plasmids in some members of *Vibrio* genus are directly correlated with many drug resistance [14,15].

Antibiotic resistance genes (ARGs) are widespread in *Vibrio*

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species, such as β-lactam resistant gene *blaTEM* [16], chloramphenicol resistant genes *catII*, *catIV* and *floR* [17,18], tetracycline resistant genes *tetA*, *tetB*, *tetD*, *tetM* and *tetS* [19,20] and streptomycin resistant genes *strA* and *strB* [21]. The intra and interspecies transmission of these ARGs may occur via three main processes: conjugation, transduction, or transformation [22].

Iron (Fe) is an essential element for the growth of pathogenic bacteria, is fundamental and necessary for establishment and replication inside a host, and is required to cause infection [23]. When iron is limiting, *Vibrio* species produces one or more small high-affinity ferric iron chelating compounds called siderophores, to acquire iron from different sources or under varied environmental conditions [24–27].

During Vibriosis infection process, virulence appear to be associated with multiple factors such as production of extracellular enzymes and toxins [28,29]. *V. alginolyticus* possesses numerous secreted proteins, including a thermolabile hemolysin and a low molecular weight ciliostatic toxin, suggesting that may play a significant role in the *Vibrio* pathogenesis in fish and shellfish [30,31].

Currently, Molecular epidemiological techniques were widely used for genetic typing of *Vibrio* species [32,33]. Among such techniques, Entero-Bacterial Repetitive Intergenic Consensus (ERIC) based genotyping and Random Amplified polymorphic DNA (RAPD-PCR) are now considered to be the most accurate methods to quickly and easily differentiate *V. alginolyticus* clones without any prior information of the gene sequence [34,35].

Hence, the present work describe the isolation and characterization of nine *V. alginolyticus* strains associated with mortality of juvenile aquacultured grooved carpet shell clam in Monastir lagoon. The epidemiological relationship between *Vibrio* isolates was determined using two DNA based fingerprinting techniques (RAPD and ERIC-PCR). The antimicrobial sensitivity, the distribution of some antibiotic resistance genes and the siderophores production were also carried out. The pathogenicity potential of the tested strains was evaluated using *R. decussatus* larva and juveniles as experimental infection models.

2. Material and methods

2.1. Bacteriological study

2.1.1. Isolation and bacterial characterization

Juvenile *R. decussatus*, coming from the mollusk hatchery of the National Institute of Sciences and Marine Technologies, were reared in cages placed on the Monastir Lagoon (35°44′N/10°49′E). Dead and moribund juvenile carpet shell clam were collected from different outbreaks occurred during the summer of 2008 and 2009. Thirty clams, ranging from 3 to 5 mm, were isolated from each outbreak. Samples were rinsed three times with artificial sterile seawater (ASSW) [36] and were crushed using sterile, autoclaved pestle and mortar in the same solution (3 ml). The homogenates were plated on thiosulfate citrate bile sucrose agar (TCBS-2% NaCl) and marine agar (Difco, France). Dominant colonies were selected and were streaked for purity on Trypticase Soy Agar plates (TSA) supplemented with 2% NaCl and incubated at 30 °C for 24–48 h for pure culture isolation and characterization.

Nine *V. alginolyticus* strains were isolated from seven different outbreaks (O1Va70, O1Va78, O2Va80, O3Va85, O4Va67, O5Va75, O6Va90, O6Va97 and O7Va112). Preliminary identification of the strains had been performed on the bases of colony morphology on TCBS (Scharlau Microbiology, Spain) supplemented with 2% NaCl, Gram nonstaining (KOH) method, cytochrome oxidase activity, motility (Mannitol-Motility agar; Pronadisa, Madrid, Spain), resistance to vibriostatic O129 (10 and 150 μg), salt requirement (growth on 0%, 2%, 4%, 8% and 10% NaCl medium) and growth at 23 and 37 °C. The species identification of *Vibrio* was performed using biochemical tests, including API 20 NE strips (bioMerieux, Marcy l'Etoile, France), and followed by a molecular characterization by targeting a housekeeping gene that encodes the heat shock protein 40 (*hsp-40*) as described previously by Nhung et al. [37].

Ability of *Vibrio* isolates to produce extracellular enzymes such as lipase, amylase, lecithinase, caseinase and Dnase was performed as described previously [38]. *Vibrio* strains were assessed for hemolytic activity on blood base agar supplemented with 5% (v/v) human blood. The strains were conserved as frozen stocks at −80 °C in tryptic soy broth (TSB; Bio-Rad, France) with 2% NaCl plus 15% (v/v) glycerol.

2.1.2. Antibiotic sensitivity testing

Susceptibility testing was performed by Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute guidelines [39] and as performed by Ottaviani et al. [28]. The following panel of antibiotic discs and concentrations were used: ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), gentamicin (10 μg), flumequine (30 μg), oxolinic acid (30 μg), streptomycin (10 μg), tetracycline (30 μg) and erythromycin (15 μg). After incubation at 37 °C for 18–24 h, findings of antibiotic resistance testing were interpreted as sensitive, moderately sensitive and resistant as recommended by CLSI [27]. *Escherichia coli* ATCC 25922 was used as a control strain.

2.1.3. Determination of minimum inhibitory concentration (MIC)

MIC's of tetracycline, flumequine, chloramphenicol, oxolinic acid and erythromycin (Sigma-Aldrich, USA) were determined by the microtitre broth dilution method according to Clinical and Laboratory Standards Institute guidelines [40]. The tested isolates were suspended in 0.85% saline to a turbidity equivalent to a 0.5 McFarland standard (1×10^8 CFU/ml) and serially diluted to obtain a concentration of 5×10^5 CFU/ml. 100 μl aliquots of the bacterial suspension were used to inoculate sterile U shaped bottom 96-well microtiter plates containing 100 μl of the tested antibiotics at a concentration equal to two times the final concentration (0.125–256 mg/l). The microtiter plates were incubated at 35 °C for 18–20 h after which they were examined for the presence or absence of growth. Well containing 200 μl of sterile broth served as negative control. No interpretive criteria were available for *V. alginolyticus* based on CLSI guide-line, the MIC values were compared to the interpretive MIC breakpoints for susceptibility among *V. cholerae* [40]. Breakpoints not available from CLSI (flumequine, oxolinic acid and erythromycin) were derived from ranges used in similar studies [41,42]. *Escherichia coli* ATCC 25922 was used for quality control in each run.

2.1.4. Antibiotic resistance genes

Genomic DNA was extracted using Wizard genomic DNA purification kit (Promega, France) according to the manufacturer's instructions. The *ermB* primer set (ermB-F 5′-AGACA CCTCGTCTAACCTTCGCTC-3'; ermB-R5′TCCATGTACTACCATGCCA CAGG-3′) was designed to amplify a 640 pb fragment of the erythromycin resistance gene [43]. The *tetS* primer set (tetS-F 5′-ATCAAGATATTAAGGAC-3'; tetS-R 5′-TTCTCTATGTGGTAATC-3′) was designed to amplify a 590 pb fragment of the tetracycline resistance gene [44]. PCR amplification was performed in 50 μl volumes containing 5x PCR buffer (Promega, France), 200 μmol/l of each desoxyribonucleotide triphosphate, 1.5 mmol/l of MgCl2, 1 U Taq polymerase (Promega, France), 1 μmol/l of each primer, and 20–30 ng of the template. The thermal cycling conditions performed with a Mastercycler from Eppendorf, were as follow: 5 min at 95 °C, followed by 30 cycles of amplification. Apart from the primer annealing temperature, each cycle consisted of