Characterization of *Vibrio Alginolyticus* Trh Positive From Mediterranean Environment of Tamouda Bay (Morocco)

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Abstract *Vibrio alginolyticus* is a halophilic *Vibrio* and is considered the most frequent species living freely in water and sediment and can survive in seawater even under starvation conditions while maintaining its virulence. Our objective in this study is to investigate the existence of virulence genes in *Vibrio alginolyticus* in Tamouda bay (Morocco). A total of 588 samples were collected during the study and analyzed. The study of cultural biochemical and molecular characteristics of strains showed an incidence of 70.2% of *Vibrio alginolyticus*. Among 412 strains of *Vibrio alginolyticus* identified eleven (2.7%) were urease and Kanagawa Phenomenon (KP) positive. To study the presence of the gene for virulence genes in ten strains of *Vibrio alginolyticus* urease positive and KP positive, we used the polymerase chain reaction (PCR). The results revealed that 70% of the strains have the *trh* gene (250 bp) but all strains are *tdh* negative. This is the first report who demonstrated the presence of *V. alginolyticus* KP positive and the implications of the *trh* gene in plankton, sediment, sea water and shellfish as a pathogen of food poisoning.

Keywords *Vibrio Alginolyticus*, Virulence Genes, Urease, Kanagawa Phenomenon, Haemolysin, PCR, *Trh*, *Tdh*, Mediterranean Sea, Morocco

1. Introduction

*Vibrio* (*V*.) is a genus of bacteria indigenous to the aquatic environment. Some bacterial species of this genus are now considered as emerging pathogens, involved in food-borne infections in humans[6], which poses a public health risk. Several studies have been conducted on the prevalence of *Vibrio* in seafood in Morocco[4,5,7,8].

*V. alginolyticus* is considered one of the most frequent species living freely in water and sediment[10] and can survive in sea water even in famine conditions while maintaining their virulence[3].

The first reports identifying *V. alginolyticus* as possessing the *trh* gene occurred in Alaska[19] and in Tunisia[2]. In addition, it has been shown that strains of *V. alginolyticus* carry the *trh* gene and the pathogenic *V. alginolyticus* strains is recognised as a potential reservoir of many known virulence genes of other *Vibrio* species in the aquatic environment which have been demonstrated to contribute to the onset of wound infections, enteric pathologies, sepsicaemia and peritonitis in humans by exposure to seawater[17,26].

The same studies have underlined the virulence of *Vibrio*.

Another study in Australia reported a case of *V. alginolyticus* in China[28] confirming reports from other countries in Europe and America[1,11,18,21,25].

This study is a follow-up investigation to the prevalence and environmental impact factor of *V. alginolyticus* in one of the Mediterranean coasts of Morocco[23], it will look more specifically at the virulence factors of *V. alginolyticus* urease positive strains isolated from the Tamouda Bay in Morocco.

2. Materials and Methods

2.1. Description of the Study Area

Tamouda Bay is located in the Mediterranean coast of Morocco, between Sebta at the North (35°54’N, 5°17’10”W) and Cap Negron at the South (35°40’N, 5°16’40”W) where the climate is typically Mediterranean. The average annual temperature is about 18°C, while the annual rainfall average ranges between 800 and 1000 mm.

2.2. Environmental Sampling

A total of four hundred and twelve (412) (142 sea water, 90 plankton, 73 shellfish and 107 sediment) samples were collected in three sampling sites at Tamouda Bay. One of the three coastal sites, located at the mouth of the Smir’s river (site 2), is described high risk while the two others (sites 1 and 3) are considered low risk. All samples were analyzed...
for the monitoring of physicochemical parameters of seawater taken from the as well as identification of Vibrio alginolyticus in water samples, plankton, shellfish and sediment. Bimonthly samples were made over a period of two years (January 2007- December 2008).

2.3. Phenotypic Identification of Bacteria

Presumptive identification was performed on the 412 strains of *V. alginolyticus* isolated by the search for oxidase, arginine dihydrolase (ADH), lysine decarboxylase (LDC) and ornithine decarboxylase (ODC) assays, and a serial growth in salt consists of a series tube of alkaline peptone water containing 2 to 10% NaCl. The identification was then continued only with oxidase positive strains, negative ADH and positive LDC by performing seeding on the API 20E commercial Kit (Biomerieux, Marcy l’Etoile, France).

2.4. Molecular Identification

Robert-Pillot and al.[22] demonstrated that amplification of the R72H fragment, for amplicons of 320 bp or 387 bp, is a powerful tool for reliable identification of *V. parahaemolyticus*. Consequently, for this study, the biochemical identification of *V. alginolyticus* strains was confirmed by the absence the sequence of r72h as described by Lee [15]. The application of this molecular study of strains biochemically identified as *V. alginolyticus* eliminates any strains of *V. parahaemolyticus* atypical for sucrose[22].

Bacterial DNA was extracted following the protocol designed for the extraction of DNA from gram positive and gram negative bacteria in the commercial kit Wizard Genomic DNA Purification Kit (Promega, France). The oligonucleotide primers relating to r72h and size of the amplicons are displayed in table 1. The PCR mixture contained 1x PCR amplification buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 1.5 mM MgCl₂, 200 mM (each) deoxyribose triphosphates, 1 mM (each) primers, 1.25 U of Taq DNA polymerase (Invitrogen), 5 ml (40 ng) of template DNA or lysed bacterial broth, and double-distilled water containing 2 to 10% NaCl. The identification was then continued only with oxidase positive strains, negative ADH and positive LDC by performing seeding on the API 20E commercial Kit (Biomerieux, Marcy l’Etoile, France).

2.5. Detection of Kanagawa Phenomenon

In order to detect the power haemolysin strains of *V. alginolyticus*, the Kanagawa phenomenon (K.P.) was studied on Wagatsuma blood agar[9]. All urease positive strains of *V. alginolyticus* (10 strains) were examined by culturing overnight in tryptic soy broth supplemented with 7% NaCl on Wagatsuma agar which contained 5% washed rabbit erythrocytes, 0.5% yeast extract, 1% peptone, 7% NaCl, 0.0001% crystal violet, and 1.5% agar (pH 7.5).

The inoculation was made by dosing 20 µl of a drop (optical density OD = 0.5) of the supernatant water tubes Alkaline Peptone (APW) in wells provided for this purpose. Results were recorded after 24 hours of incubation at 37°C. A clear zone of haemolysis around the sample on Wagatsuma blood agar plate was considered a positive Kanagawa reaction (KP+). However, strains of *V. alginolyticus* which were confirmed to be positive using the urease and which demonstrated a positive KP result were subjected to the detection of virulence genes by PCR.

2.6. Detection of Virulence Genes

Purification of plasmid DNA

In order to confirm the presence of structural trh and tdh genes in *V. alginolyticus* strains, we used *V. parahaemolyticus* strains as a positive control for both of these genes. Bacterial DNA was extracted following the protocol designed for the extraction of DNA from gram positive and gram negative bacteria in the commercial kit Wizard Genomic DNA Purification Kit (Promega, France).

Oligonucleotide primers

The oligonucleotide primers specific for the trh and tdh genes and size of the amplicons following PCR amplification are described in table 1.

**Table 1.** Sequences of oligonucleotide primers, amplicon size used for the PCR amplification

| Target gene | amorce Primer sequence | Amplicon size (bp) | Reference |
|-------------|------------------------|-------------------|-----------|
| r72h        | VP32: CGAATCTGTCCCTTTTCCTGC | 320 or 387 | [15] |
|             | VP33: TGGGAAATTCCGATAGGGGTGTTAACC | | |
| trh         | L.trh: GGCTCAAAAATGTTAAGCG | 250 | [8] |
|             | R.trh: CATTGCCGCTCTCATATGC | | |
| tdh         | L.tdh: CCACATCGCTCTCTCATATGC | 373 | [8] |
|             | R.tdh: CCAAAATACATTTTACTTGG | | |

r72h = specific sequence of *V. parahaemolyticus*; trh = thermostable-related direct hemolysin; tdh = thermostable direct hemolysin; bp = base pair.
Amplification of trh and tdh gene for PCR
The amplification was optimized in a 50 µl reaction consisting of 0.5 µg of purified genomic DNA of V. alginolyticus strains, 1 µM of each of the oligonucleotide primers for trh (1.2µl of each of the primers from a 20 µM stock suspension)(Sigma), 5 µl of a 10 X PCR reaction buffer (10 X buffer consisted of 500 µM Tris-Cl, pH 8.9, 500 mM KCl) and 4 µl MgCl2 (4 mM) (Invitrogen); final concentration of 1x), 200 µM of each of the dNTPs (4 µl from a 10 mM stock dNTP) (Promega-Madison wi USA), 0.6 units AmpliTaq DNA polymerase (Invitrogen) and an appropriate volume of sterile MilliQ water (Millipore).

The PCR amplification was performed in a DNA thermal cycler (Bio-Rad, DyadDisciple) using the following temperature-cycling parameters: initial denaturation at 94°C for 5 min followed by 30 cycles of amplification; each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and primer extension at 72°C for 1 min. Following the amplification cycles, samples were kept at 72°C for 10 min to allow final extension of the incompletely synthesized DNA.

PCR products (10 µl each) were separated by agarose gel electrophoresis in a 2% agarose gel (Invitrogen) run at 75V for 1.5h in 1xTris-acetate-EDTA. Amplification products were visualized by ethidium bromide staining and visualized and photographed using a UV transilluminator (Vilber Lourmat, Germany).

3. Results and Discussion
A total of 588 samples were collected during the study, of which 70.24% were found to be positive for the incidence of V. alginolyticus. The highest incidence was found in water (34%) followed by sediment (26%), plankton (22%) and shellfish (18%).

Furthermore, our study showed a prevalence of V. alginolyticus from 70% in shellfish analyzed, a prevalence highly superior to the 50%[5] and 8.2%[8] reported for this species for shellfish marketed in Morocco. Among 412 strains of Vibrio alginolyticus identified, eleven (2.7%) were urease and KP positive. Moreover, all ten strains assumed to be V. alginolyticus were confirmed by the absence of the gene r72h. PCR analyses revealed 70% of the samples positive for trh (250 bp). Figure 1 shows that only strains Va2, Va4, Va6, Va7, Va8, Va9 and Va10 were trh positive, while strains Va1, Va3 and Va5 were trh negative. However, all strains are tdh negative.

Moreover, trh positive strains showed a zone of haemolysis with a diameter greater than 11.2 mm. While trh negative strains have a diameter between 2 and 5.2 mm (table 2).

Being an autochthonous marine bacterium, V. alginolyticus is probably subjected to a high level of recombination with the diverse, closely related bacterial strains populating marine environments. Marine environments provide a habitat where Vibrio can be exposed to high levels of gene transfer by transduction[14], and consequently, putative transfers of virulence factor genes like trh and tdh can occur between marine bacteria.

| Strains | Ecological origin | ADH | LDC | ODC | Grown in NaCl 2 – 10 % | Urease test | Pr72H Phenomenon / Diameter (mm) | trh | tdh | Results identifications |
|---------|------------------|-----|-----|-----|------------------------|------------|--------------------------------|-----|-----|-------------------------|
| Va 1    | Sediment Site 3  | -   | +   | -   | +                      | +         | + / 3.1                        | -   | -   | V. alginolyticus        |
| Va 2    | Sea water Site 2 | -   | +   | +   | -                      | +         | + / 21.8                       | +   | -   | V. alginolyticus        |
| Va 3    | shellfish        | -   | +   | +   | -                      | +         | + / 5.2                        | -   | -   | V. alginolyticus        |
| Va 4    | Sea water Site 2 | +   | -   | -   | +                      | -         | + / 14.7                       | +   | -   | V. alginolyticus        |
| Va 5    | Plankton Site 3  | -   | +   | -   | +                      | -         | + / 2.0                        | -   | -   | V. alginolyticus        |
| Va 6    | Plankton Site 3  | -   | +   | -   | +                      | -         | + / 19.3                       | +   | -   | V. alginolyticus        |
| Va 7    | Plankton Site 3  | +   | -   | +   | +                      | -         | + / 11.2                       | +   | -   | V. alginolyticus        |
| Va 8    | Sediment Site 2  | +   | -   | +   | +                      | +         | + / 16.9                       | +   | -   | V. alginolyticus        |
| Va 9    | Sediment Site 2  | -   | +   | -   | +                      | +         | + / 24.4                       | +   | -   | V. alginolyticus        |
| Va 10   | Sea water Site 1 | -   | +   | -   | +                      | -         | + / 21.1                       | +   | -   | V. alginolyticus        |

API 2 0E: BioMerieux, Marcy l’étoile, France ADH:arginine dihydrolase; LDC : lysine decarboxylase; ODC: ornithine decarboxylase ; cm: centimeter
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4. Conclusions

This study pioneers the demonstration of the presence of KP positive and trh V. alginolyticus in Morocco. The presence of pathogenic V. alginolyticus strains in seawater, plankton, sediment, and shellfish represent a risk of infection following exposure and indicates the potential sanitary risk associated with the presence of V. alginolyticus KP positive strains and those carrying the trh gene as pathogens implicated in cases of food poisoning[13].

To conclude, our results suggest that a long-term monitoring program should be initiated to detect pathogenic V. alginolyticus KP positive strains and those carrying the trh gene in the aquatic environment during the warm summer months, when concentrations of this bacterium in Tamouda Bay are thought to be at their highest[23].
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