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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 *trh* gene in Morocco. These findings indicate the potential sanitary risk associated with the presence of *Vibrio alginolyticus* KP positive and the implications of the *trh* gene in plankton, sediment, sea water and shellfish as a pathogen of food poisonning.

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 contributes to the onset of wound infections, enteric pathologies, septicaemia 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℃, 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

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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 $MgCl_2$, 200 mM (each) deoxynucleoside 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 treated with 0.1% diethylpyrocarbonate to make a final volume of 50 ml and was then subjected to 35 PCR cycles in a programmable temperature cycler (Bio-Rad, DyadDisciple). The parameters for the amplification cycles were denaturation for 1 min at 94℃, annealing of primers for 1 min at 60℃, and primer extension for 1 min at 72℃. After the last cycle, the PCR mixtures were incubated for 10 min at 72℃.

PCR-amplified DNA was detected by agarose gel electrophoresis in 2% agarose gels (invitrogen). Ten µl each of the amplification mixtures was subjected to electrophoresis and ethidium bromide staining. The specific amplified DNA fragments were visualized by UV illumination.

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℃. 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 demontrated 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*	CGAATCCTTGAACATACGCAGC	320 or 387	[15]
	VP33	TGCGAATTCGATAGGGTGTTAACC		
trh	*L.trh*	GGCTCAAAATGGTTAAGCG	250	[8]
	R.trh	CATTTCCGCTCTCATATGC		
tdh	*L.tdh*	CCATCTGTCCCTTTTCCTGC	373	[8]
	R.tdh	CCAAATACATTTTACTTGG		

r72= 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 $MgCl_2$ (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.

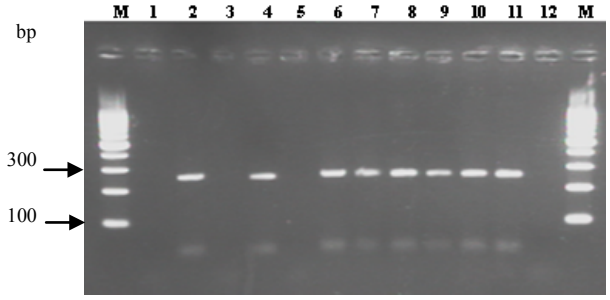

Figure 1. Agarose gel electrophoresis of the products obtained after PCR amplification of the *trh* genes. Lanes: 1, Va1; 2, Va2 ; 3, Va3; 4, Va4; 5, Va5; 6, Va6; 7, Va7; 8, Va8; 9, Va9; 10, Va10; 11, Vp ; 12, T-. M indicates a 100-bp size ladder

Table 2. Biochemical and molecular identification of strains

Strains	Ecological origin	ADH	LDC	ODC	Grown in NaCl 2 – 10 %	Urease test	*Pr72H*	Kanagawa 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

The presence of a *trh* gene actively expressed in a *V. alginolyticus* strain supports the hypothesis that this gene is transferred among *Vibrio* and this shows that *V. alginolyticus* often possessed homologues of virulence genes of *V. parahaemolyticus* and *V. cholerae*, suggesting that *V. alginolyticus* can be a reservoir for these genes in the aquatic environment[28]. The practical implications of these results are that detection of the *trh* gene in mixed cultures, such as broth enrichments or nucleic acid extracts of seafood or environmental samples, does not always imply that pathogenic *V. parahaemolyticus* is present.

Many *Vibrio* species are pathogenic for humans and/or marine vertebrates and invertebrates, with the virulence mechanisms reflecting the presence of enterotoxin haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and/or haemagglutinins.

Haemolysin is an exotoxin that attacks blood cell membranes and causes cell rupture. Haemolysis, which results from the lyses of erythrocyte membranes with the liberation of haemoglobin, consists of β-haemolysis, i.e. the complete degradation of haemoglobin, and α-haemolysis, i.e. the incomplete degradation of haemoglobin. Haemolysins are produced by many different species of bacteria including Escherichia coli, Pseudomonas aeruginosa and vibrios. In most cases, epidemiological and experimental evidence suggests that haemolysins are involved in disease pathogenesis[16,24,27].

In fact, studies have shown that the *trh* gene had significant nucleotide sequence homology with the *tdh* gene. The amino acid sequences of the haemolysin subunits deduced from the nucleotide sequences of the *trh* gene and t*dh* gene were homologous and contained the two cysteine residues to form an intrachain bond at the same positions[20]. Moreover, they have similar biological, immunological and physicochemical characteristics[12], which explains that the genes *tdh* and *trh* may have had a common ancestor and may have evolved by a single base change so they can maintain the basic architecture of the molecules.

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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