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Occurrence of Pathogenic *Vibrio* Species in Tamouda Bay (Morocco)

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Abstract Samples of seawater, plankton, shellfish and sediments collected from coastal sites located in Mediterranean (Northern Morocco) were examined for the presence of pathogenic *Vibrio* species and the occurrence of pathogenic strains. The isolation of the microorganisms was performed by using a standard method. A biochemical protocol was applied for the identification of the isolates while polymerase chain reaction (PCR) was used to confirm the identification of the strains and to detect the virulence genes. Results showed that 14.8%, 8.1%, 31.3% and 28.5% of seawater, plankton, shellfish and sediments samples respectively contained pathogenic *Vibrio* species. The most frequently isolated pathogenic *Vibrio* species was *V. parahaemolyticus.* Out of 139 strains of *V. parahaemolyticus* isolated, 23.7% had *trh* gene while none was positive for *tdh* gene. Among *V. parahaemolyticus trh* positive, 31 (93.9%) strains were urease positive. All 32 strains of *V. cholerae* isolated during this study were non-O1 non-O139 and negative for the presence of *ctx*A, *ctx*B and *tcp*A genes.

Keywords *Vibrio* spp., Pathogenic *Vibrio*, Mediterranean sea, Morocco

1. Introduction

Vibrio is a Gram-negative genus of facultative anaerobes straight or curved rods, motile by one or more polar flagella, that give a positive oxidase test, grow on thiosulfate citrate bile-salt sucrose agar. Most species are sensitive to the Vibriotactic agent O/129. Sodium stimulates growth of all species and required for most species[1].

Vibrios are normal inhabitants of aquatic environments, being very common in marine and estuarine habitats and on the surface and in the intestinal contents of marine animals [2, 3]. 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[4, 5]. Among these species, *V. cholerae* is the most important, as it is associated with epidemic and pandemic diarrhoea outbreaks in many parts of the world[6]. More than 200 serogroups of *V. cholerae* are known, but only serogroups O1 and O139 cause epidemic and pandemic cholera[5]. The pathogenesis of cholera is a complex process and involves a number of factors which help the pathogen to reach and colonize the epithelium of the small intestine and produce the enterotoxin that disrupts ion transport by intestinal epithelial cells. In *V. cholerae*, the major virulence genes appear to exist in clusters, and there are at least two regions of the *V. cholerae* chromosome in which genes encoding virulence factors are clustered[7, 1, 8, 9, 10]. These include the *CTX* element, which has now been shown to be the genome of a filamentous bacteriophage[11], and the *TCP*-accessory colonization factor (*ACF*) gene cluster, referred to as the *TCP* pathogenicity island[12]. However, other species of *Vibrio* (including *V. parahaemolyticus* and *V. vulnificus*) capable of causing disease in humans have received greater attention in the last decade[2]. Although there are many strains of *V. parahaemolyticus*, only those that produce the thermostable direct hemolysin (*TDH*) and/or the thermostable related hemolysin (*TRH*) have the ability to cause gastroenteritis[13], and almost all the strains isolated from clinical samples have either or both genes (*tdh* and *trh*) which encode the respective hemolysins. *V. vulnificus* is a human pathogen that is highly invasive, causing fulminant pulmonary septicemia, with mortality rates as high as 75%, one of the highest death rates of any foodborne disease[14, 15]. *V. vulnificus* infection is most lethal in individuals who have a preexisting chronic illness, are immunocompromised or have preexisting liver disease [14, 15, 16]. *V. vulnificus* can express various virulence-associated factors, including capsular polysaccharides, lipopolysaccharides, extracellular hemolysin/cytolysin, metalloprotease, pili, Xagella, siderophore, and repeats-in-toxin proteins[15, 17].

In an attempt to contribute to the clarification of the ecological relationships between sea environment and

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Vibrio in the Mediterranean areas, we carried out a two years study to investigate, by both culture and polymerase chain reaction (PCR) the presence of potential pathogenic *Vibrio* species (*V. vulnificus*, *V. parahaemolyticus* and *V. cholerae*) along Tamouda Bay located in Mediterranean coast of Morocco. In view of the increasing evidence supporting the role of halophylic *Vibrios* as both human pathogens and a reservoir of *Vibrio* virulence genes[18, 19]. Another aspect of this work was to investigate the presence of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* pathogenicity-associated genes in *Vibrio* strains isolated.

2. Materiels and Methods

2.1. Description of the Study Area and Environmental Sampling

Samples were collected bimonthly from January 2007 to December 2008 in three sampling sites of Tamouda Bay. The locations of the sites were determined by use of the global positioning system (Fig. 1). For each field visit, four ecological types of samples were collected, namely, seawater, planktons, shellfish and sediments.

Seawater samples (2L) were collected from a boat 1m below the surface using sterilized plastic bottles. Planktons were collected by dragging the water horizontally, at a depth of about 1m, with 200-µm-mesh plankton net[20]. Shellfish samples were purchased from local fisherman, while the sediments were collected from the surface of the coast using sterile plastic pots. After collection, the samples were transported immediately to the laboratory in insulated coolers with frozen gel-packs to maintain a temperature at around 4°C.

2.2. Detection of Potential Pathogenic *Vibrio* Species by a Conventional Method

In the laboratory, shellfish were immediately removed from the bags, washed and scrubbed under running potable water to remove debris and attached algae; dead mussels or those with broken shells were discarded.

Approximately 5 specimens were opened aseptically with a sterilized scalpel. Meat and shell liquid were collected in a sterile jar, cut with scissors and mixed thoroughly. Then, 25 grams liquid and meat were transferred into sterile plastic bags with 250 mL of Alkaline Peptone Water (APW; 1% [wt/vol] peptone, 2% [wt/vol] sodium chloride; pH 8.2) to obtain a 1:10 dilution and subjected to further homogenization for 60 s using a stomacher.

All the collected volume (2L) of each water sample was concentrated by filtration through a 0.22 µm-pore-size filter (Millipore Corp., Bedford, MA, USA) with a vacuum pump, and filters were subsequently placed in 20 ml of APW to obtain a final concentrated volume corresponding to 100x.

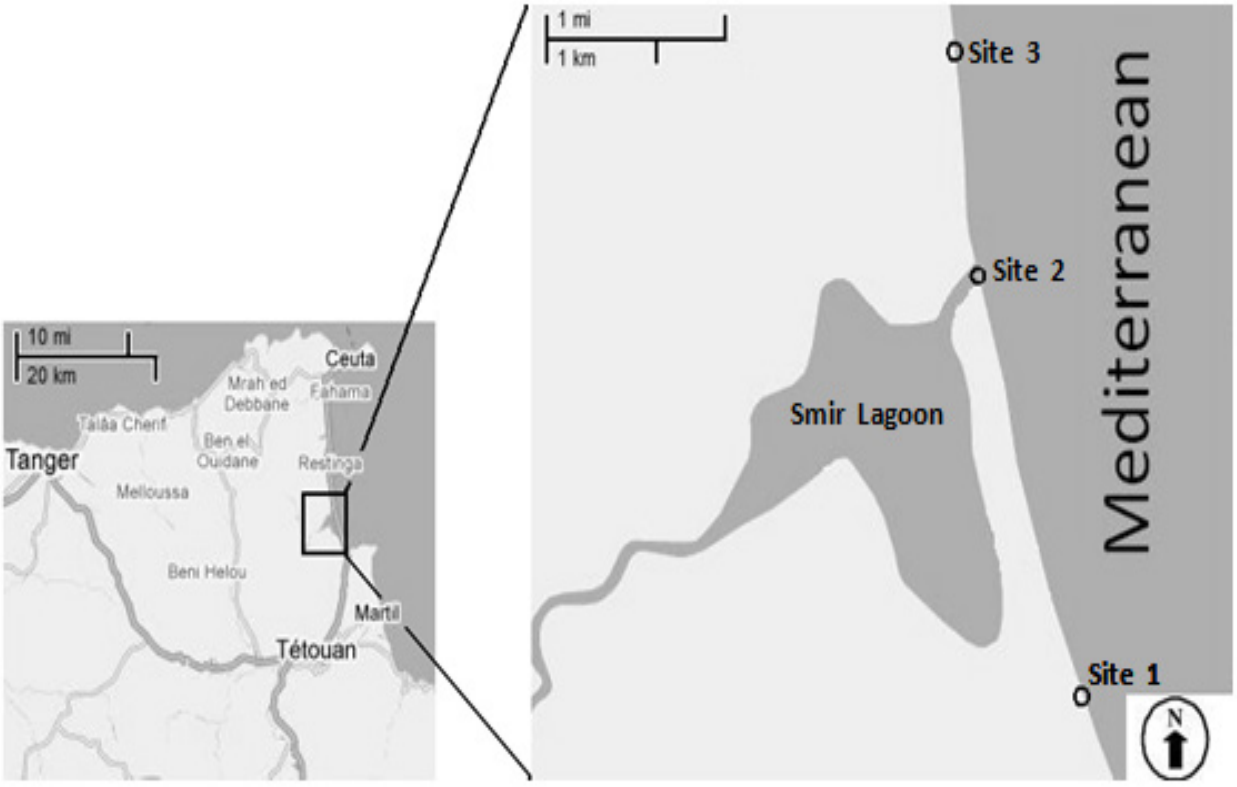

Figure 1. Map showing the geographical location of three sampling sites (sites 1: 35° 41' 184"N, 005° 19' 258"W; site 2: 35° 41' 402"N, 005° 18' 226"W and site 3: 35° 40' 885"N, 005° 18' 760"W)

Regarding sediment samples, 25g were inoculated into 250 mL of Alkaline Peptone Water (APW) and mixed using a stomacher for 60 s, while planktons were used directly for *Vibrio* spp. detection. Inoculated APW was incubated at 37°C for 16-18 h. The enrichment broth was then sub-cultured onto Thiosulfate Citrate Bilesalt Sucrose (TCBS) agar and incubated at 37°C for 18-24h.

From each TCBS The suspected colony types (yellow and green) were picked out, streaked onto nutrient agar plus 2% NaCl to obtain pure cultures, screened for cytochrome oxidase, and examined for NaCl requirement (0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%). Other morphological, biochemical and cultural tests carried out were Gram staining, catalase reaction and aminoacids decarboxylase reaction. Then, the suspected colonies were submitted to phenotypic characterization by API 20 E (BioMerieux, France) employing 1% sterile saline as inoculum diluent.

Serotyping of V. cholerae isolates was performed by the slide agglutination test using commercial polyvalent anti-O1 and anti-O139 antisera (Denka Seiken Co. Ltd, Tokyo, Japan).

2.3. Molecular Identification of Pathogenic *Vibrio* Species by PCR

The biochemical identification of isolated strains identified as pathogenic *vibrio* species was confirmed by PCR on the purified DNA.

DNA extraction: The extraction and purification of DNA was carried out using a previously described method[21] wich was modified as follows. In fact, cells either from 18 h cultures in nutrient agar containing 2% NaCl were harvested or resuspended in 300µL of TENa buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Lysis was accomplished by the addition of 50µl of 10% sodium dodecyl sulfate (SDS) and 10µl of pronase. Before use, pronase was self-digested at 37°C for 30 min followed by another incubation at 60°C for the same duration (30min) to remove any contaminating DNases. An equal volume of isoamylic phenol/chloroform was added to the cell lysate and mixture was shaken to equilibrate the phenol and aqueous phases which were then separated by centrifugation for about 10 min at 15000 rpm. Two volumes of cold absolute ethanol were added to the aqueous phase to precipitate nucleic acids.

After another centrifugation at 15000 for 10 min, the DNA precipitate was washed with 70% ethanol and air-dried. It was then dissolved in 100µl of TE containing 0.002% of RNase. Purity was calculated following determination of A260/A280 ratios, and DNA concentrations were obtained from the A260 values.

Polymerase Chain Reaction: The presence of *V. cholerae, V. vulnificus* and *V. parahaemolyticus* was also identified by PCR. Molecular identification of *V. cholerae* and *V. vulnificus* was carried out targeting the *16S-23S* rDNA[22] and the cytotoxin-hemolysin gene (*Hly*)[23] respectively, while all strains identified biochemically as *V. parahaemol yticus* or *V. alginolyticus* were analyzed targeting pR72H fragment[24] and regulatory gene toxR[25].

The presence of *ctx*A and *ctx*B encoding subunits A and B of cholera toxin were determined by using simplex PCR assays followed the methods of Fields et al. and Olsvik et al. respectively[26, 27], while the amplification of the *tcp*A gene encoding the toxin-coregulated pilus (*TCP*) was performed using PCR conditions previously described[28]. PCR amplification of the *tdh* and *trh* genes encoding respectively the thermostable direct hemolysin (*TDH*) and the thermostable related hemolysin (*TRH*) of *V. parahaemolyticus* was carried out according to Bej et al.[29]. The various oligonucleotide sequences used in this study are shown in Table 1. PCR products were electrophoresed in a 1.6% agarose gel, stained with ethidium bromide and visualized under UV light.

Table 1. Primers used in the study

Species	Target genes	Primers	Sequences 5'-3'	Amplicon size (bp)
V. parahaemolyticus	*pR72H*	*VP32*	CGAATCCTTGAACATACGCAGC	320 or 387
		VP33	TGCGAATTCGATAGGGTGTTAACC	
	toxR	*ToxR 4*	GTCTTCTGACGCAATCGTTG	368
		ToxR 7	ATACGAGTGGTTGCTGTCATG	
	tdh	*L-tdh*	GTAAAGGTCTCTGACTTTTGGAC	269
		R-tdh	TGGAATAGAACCTTCATCTTCACC	
	trh	*L-trh*	TTGGCTTCGATATTTTCAGTATCT	500
		R-trh	CATAACAAACATATGCCCATTTCCG	
V. cholerae	*16S-23S*	*VC-F*	TTAAGCGTTTTCGCTGAGAATG	295 to 310
		VCM-R	AGTCACTTAACCATACAACCCG	
	ctxA	*CTX2*	CGGGCAGATTCTAGACCTCCTG	564
		CTX3	CGATGATCTTGGAGCATTCCCAC	
	ctxB	*CTX7*	GGTTGCTTCTCATCATCGAACCAC	460
		CTX9B	GATACACATAATAGAATTAAGGATG	
	tcpA	*72F*	CACGATAAGAAAACCGGTCAAGAG	451 or 620
		477R	CGAAAGCACCTTTCACGTTG	
		647R	TTACCAAATGCAACGCCGAATG	
V.vulnificus	*Hly*	*Vv-1*	CGCCGCTCACTGGGGCAGTGGCTG	388
		Vv-3	CCAGCCGTTAACCGAACCACCCGC	

3. Results

Table 2. Incidence of positive samples for pathogenic *Vibrio* species in different environmental types

Environmental type of samples	Number of analyzed samples	Incidence of positive samples	Incidence of pathogenic *Vibrio* species	
			V. cholerae	*V. parahaemolyticus*
Seawater	236	35 (14.8%)	10 (4.2%)	25 (10.6%)
planktons	198	16 (8.1%)	0(0%)	16 (8.1%)
Shellfish	67	21 (31.3%)	3 (4.5%)	18 (26.9%)
Sediments	130	37 (28.5%)	12 (9.2%)	25 (19.2%)
Total	n = 631	109 (17.3%)	25 (4%)	13.3%)

Table 3. Incidence of positive samples for pathogenic *Vibrio* species in the three simpling sites

simpling site	Numbre of analyzed samples	Incidence of positive samples	Incidence of pathogenic *Vibrio*	
			V. cholera	*V. parahaemolyticus*
site 1	209	11 (5.3%)	6 (2.9%)	5 (2.4%)
site 2	234	88 (37.6%)	17 (7.3%)	71 (30.3%)
site 3	188	10 (5.3%)	2 (1.1%)	8 (4.3%)

Of 631 samples examined, 4% were positive for *V. cholerae* and 13.3% for *V. parahaemolyticu*s. The incidence of *V. parahaemolyticus* was more important than that of *V. cholerae* in all environmental types of analyzed samples. Nevertheless, no strain of *V. vulnificus* has been detected (Table 2).

The highest incidence of *V. parahaemolyticus* was found in shellfish (26.9%), while those *of V. cholerae* was recorded in sediments (9.2%) (Table 2). Results showed also that the highest incidence of pathogenic *Vibrio* species was consistently recorded in site 2 (Table 3).

In this study 170 strains were isolated. The species most frequently found was *V. parahaemolyticus* (81.8%), followed by *V. cholerae* (18.2%). It was also noted that 24 strains from those biochemically identified as *V. alginolyticus* were confirmed as V. parahaemolyticus by PCR method.

Out of 139 strains of *V. parahaemolyticus* isolated, 33 (23.7%) showed positive results for the presence of the *trh* gene while none has been positif for *tdh* gene. Among *V. parahaemolyticus TRH* positive, 31 (93.9%) strains were urease positive. All 32 strains of *V. cholerae* isolated were non-O1 non-O139 and negative for the presence of *ctx*A, *ctx*B and *tcp*A genes.

4. Discussion

Despite their clinical and epidemiological relevance and their elevated prevalence in the aquatic environment, most mediterranean countries do not routinely determine the presence of pathogenic *Vibrio* species in clinical, environmental and food sources. In two years study of pathogenic *Vibrio* species were detected in marine environment of Tamouda bay. Values encountered were highest than those of the same species found by Ripabelli et al. where of the total of sixty-two samples of *Mytilus galloprovincialis* (mussels) harvested from approved shellfish waters in the Adriatic Sea in Italy (in the Mediterranean), 1.6% were positive for NCV with the same incidence (1.6%) of positive samples for *V. parahaemolytic us*[30]. But in the same study 17.7% of samples were positive for *V. vulnificus*[30] while in our study any sample of shellfish was positive for this species. The incidence of *V. parahaemolyticus* in shellfish found was also highest than those found by Bouchriti *et al.*[31] and Cohen *et al.*[32] where just 5% and 10% where respectively positive for this species.

Moreover, out of 109 samples positive for pathogenic *Vibrio* species (*V. cholerae* and *V. parahaemolyticus*), 88 (80.7%) were collected from site 2. This may be explained by the geographic position of this site. In fact, it is located at the point of communication between Smir Lagoon and Mediterranean Sea through an opening of more than 20 meters wide, as well as in addition of Oued Smir's waters; Smir lagoon receives also, through small channels, the domestic waters of M'diq city. Therefore, this site is very rich in nutrients[33].

Is also noted that the incidence of positive samples in shellfish and sediments were highest than those in seawater and plankton. This may be explained by the capability of shellfishes to filtrated seawater and to concentrate the bacteria content in both seawater and planktonin their liquor[34]. Moreover, a lot of studies have showed that the sediments constitute a reservoir of *Vibrio* spp. which explains the high incidence of pathogenic *Vibrio* in this type of simples[35, 36, 37].

In this study 23.7% of *V. parahaemolyticus* strains isolated showed positive results for the presence of the *trh* gene while none has been positive for *tdh* gene. This is in agreement with the literature. In fact, it has been reported that more than 90% of clinical *V. parahaemolyticus* isolates but less than 1% of food or environmental strains possess

tdh[38, 39, 40]. Moreover, the result concerning occurrence of *trh* gene was similar than that obtained by a previous study reporting that the *trh* gene was present in 4/20 (20%) of *V. parahaemolyticus* strains isolated from environmental samples collected from the coast of Seto-Inland Sea in Japan[41]. However, it is in contradiction with the results obtained in most previous studies, showing that just 1% to 5% of environmental isolates possess the *trh* gene[42, 43, 44].

Of special interest, among *V. parahaemolyticus* strains *trh* positive isolated in our study, 94% were urease positive, this is in total accordance with the results found by DePaola et al[45] and Suthienkul et al.[46] where 97% and 100% respectively of isolated pathogenic *V. parahaemolyticus* which were urease positive possessed a *trh* gene. This indicates that urease production by *V. parahaemolyticus* strains strongly correlates with the possession of the *trh* gene. Thus, the urease-positive phenotype of *V. parahaemolyticus* can be considered an indication of virulent (*trh*-possessing) *V. parahaemolyticus* strains in clinical diagnosis.

It was also noted that out of 204 strains biochemically identified as *V. alginolyticus* 24 (12%) were confirmed as *V. parahaemolyticus* by PCR method. It is therefore evident that the PCR technique provides greater specificity than the phenotypic methods to differentiate these two species of *Vibrio*. In fact, the specificity and sensitivity of PCR compared to the conventional method for the identification of pathogenic Vibrios have been reported by several studies[32, 47, 48, 49, 50].

5. Conclusions

In conclusion, the results obtained showed that pathogenic *Vibrio* species were detected in shellfish and environmental samples collected from Tamouda bay. Consequently, they could also be present in seafood products in other mediterranean coast. Finally, our results suggest that a long-term monitoring program should be initiated to detect pathogenic *vibrio* species isolates in the environment, especially during the warm summer months when concentrations of this bacterium are thought to be at their highest.

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