Detection and differentiation of *Vibrio vulnificus* in seawater and plankton of a coastal zone of the Mediterranean Sea

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Abstract

*Vibrio vulnificus*, a human and animal pathogen, is present in low numbers in the Mediterranean Sea. Seawater and plankton samples were collected from a marine coastal zone of the Straits of Messina in the Mediterranean Sea (Italy) in order to investigate *V. vulnificus* as free-living (>0.2 µm) and associated with small (>64 µm) and large plankton (>200 µm) utilizing cultural and molecular techniques. Characteristic colonies, grown on thiosulfate, citrate, bile salts and sucrose agar plates, were identified using a biochemical protocol system. A PCR assay was used to confirm isolates and to directly detect *V. vulnificus* in environmental concentrated samples. Specific primers were used to target the structural cytotoxin/hemolysin gene and the variable regions of 16S rRNA species-specific for *V. vulnificus*. In addition, a tri-primer PCR of 16S rRNA was used for the differentiation of *V. vulnificus* strains. Direct detection in marine samples was more frequent than isolation of culturable forms. All isolates were assigned to *V. vulnificus* biotype 1, 16S rRNA type B. These results confirm the low incidence of *V. vulnificus* in Mediterranean coastal waters. The isolation of cultivable forms is limited to the warmest months. 16S rRNA primers were the most sensitive molecular tool as they allowed detection of *V. vulnificus* in 79.1% of samples. Due to the low incidence of *V. vulnificus* in the Mediterranean coastal environment, its detection requires a molecular approach. The occurrence of *V. vulnificus* as plankton-associated confirms the role of plankton as a potential reservoir for this pathogen.

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Keywords: *Vibrio vulnificus*; Plankton-associated vibrios; PCR assay; Cytolysin primers; 16S rRNA primers

1. Introduction

*Vibrio vulnificus* is an etiologic agent in severe human infection acquired through wounds or contaminated seafood. The strains belonging to this species are divided into three biotypes according to their different biochemical and biological properties [24]. Biotype 1 strains are pathogenic for humans, exhibit several immunologically distant lipopolysaccharide types and are indole-positive; indole-negative strains, biotype 2, appear to be virulent for both humans and eels [6]. A third biotype, indole-positive, causing wound infections and bacteremia in people handling St. Peter’s fish (*Tilapia* spp.), has been isolated in Israel [7]. Among them, biotype 1 strains are most frequently isolated from clinical specimens. Opportunistic infection in susceptible individuals typically causes mortality within 24–48 h of exposure [30,35]. *V. vulnificus* is a major bacterial cause of mortality associated with food-borne diseases, and it results in the highest death rate of any causative agent [42]. Diseases associated with *V. vulnificus* infection have been found to be present in two patterns [8]. In one, primary septicemia occurs in individuals with chronic liver disease shortly after eating raw oysters, with a mortality rate of over 50%. In the other pattern, wound infections are incurred via exposure to seawater or handling seafood products, with a death rate of approximately 25% [17]. *V. vulnificus* infections have been reported in the USA [18], Europe [12] and Asia [11,36]. Very scanty cases of *V. vulnificus* infections have been reported in Mediterranean zones.
V. vulnificus has been detected in coastal and estuarine environments throughout the world [22,32,33]. During warm weather, when the sea temperature is high, greater frequency of infection has been observed [41]. Shellfish may constitute one of the most hazardous foods if consumed raw or undercooked, as they can accumulate a high number of microorganisms from the overlying water. Mollusks possess an intense filtration activity that enables them to retain or to eliminate contaminating bacteria. The quality of the growing waters is therefore a very important factor. Bacterial fecal indicators are useless in predicting the occurrence of vibrios because the time of accumulation and of retention of these bacteria is different with respect to that of Vibrio spp. [26].

The enumeration, isolation and identification of V. vulnificus in environmental samples are laborious and inaccurate. Often culture is negative as a consequence of the presence of viable but unculturable forms [13].

Probes based on the cytolysin gene of V. vulnificus have been developed to identify presumptive V. vulnificus recovered from culture media and from environmental samples [31]. The possible loss or rearrangement of these non-essential genes can lead to a false-negative result. For this reason, alternative methods based on 23S rRNA gene (rDNA) [4] and 16S rRNA gene have been developed for molecular detection of V. vulnificus. Recently, in order to differentiate V. vulnificus strains into two variants (type A and B), a PCR assay was developed [23].

In the present study, the recovery of V. vulnificus in seawater and plankton samples collected from a coastal zone of the Mediterranean Sea was studied by conventional cultural methods and by combining a culture-based approach with a DNA-based technique (PCR). Specific primers enabled rapid and reliable identification of the presumptive isolates. To overcome the time of isolation and identification of presumptive isolates, we simultaneously carried out direct detection of V. vulnificus genes in environmental samples using two different targeting regions in a PCR assay.

2. Materials and methods

2.1. Sampling area and treatments of samples

Monthly field sampling was carried out during the warmest months of the years 2002 and 2003, from March to October. Seawater and plankton samples were collected at one station located in the Straits of Messina, ca. 50 m from the coast (lat. 38°25′21″N, long. 15°60′23″E) in the same area routinely monitored since 2001. Details of sampling and samples treatment for the collection of bacteria as free-living, associated with small and large plankton are given elsewhere [29].

2.2. Plankton analysis

Aliquots of samples were preserved in 4% (v/v) phosphate-buffered saline formaldehyde. Plankton samples were randomly subsampled using a 5-ml Stemple pipette and enumerated by counting at least 200 individuals of the most abundant zooplankter. When plankton was less abundant than 100 individuals, the entire sample was counted. Zoo-plankton was identified to order for amphipods, appendicularians, cladocerans, chaetognates, copepods, copepod nauplii, eciilates, euphausiids, molluskan pteropoda, polychaetes, rotifers and tinninids.

2.3. Culture assays

Before inoculating, large plankton samples were homogenized three times for 1 min in a glass homogenizer at 130 g. The abundance of Vibrio spp. was obtained by inoculating aliquots of concentrated samples onto thiosulfate-citrate-bile salts-sucrose (TCBS, Oxoid) agar, incubated at 37°C for 24 h, according to suggestions by Pfeffer et al. [37].

To isolate V. vulnificus strains, aliquots of seawater, small and large plankton samples were inoculated in alkaline peptone water (pH 8.4) supplemented with polymyxin B (APWP) for 18–24 h of aerobic incubation at 37°C. Turbid cultures were streaked onto plates of TCBS agar (Oxoid) incubated at 37°C for 24–48 h and CPC agar (cellobiose, polymyxin B, colistin) [27] incubated at 40°C for 24 h.

The isolates were identified by a protocol of cultural and biochemical characteristics [1,16,28]. Conventional biochemical tests were carried out in parallel with reference strains V. vulnificus ATCC 27562 biotype 1 and V. vulnificus ATCC 33149 biotype 2.

2.4. Molecular assays

PCR assays were performed to confirm V. vulnificus strain identification, to differentiate them in type A and B 16S rRNA and to directly detect V. vulnificus genes in environmental samples.

2.4.1. DNA extraction from V. vulnificus strains

Colonies of presumptive V. vulnificus isolated from TCBS and CPC agar plates were picked up, suspended in 100 µl of filtered distilled water and bacterial cells were collected by centrifugation at 11 000 g for 15 min at 4°C. The pellet was suspended in 100 µl of filtered and autoclaved water and boiled for 10 min. The cell lysates (10 µl) were used as template in the PCR assays immediately after extraction or following storage at −80°C.

2.4.2. DNA extraction of V. vulnificus genes from environmental samples

PCR analysis was performed on the same seawater and plankton samples used for cultural analysis. An aliquot (1 ml) of each concentrated sample was centrifuged at...
2.4.3. Oligonucleotide primers and PCR conditions

Primers used to detect and differentiate *V. vulnificus* strains are reported in Table 1.

TABLE 1

<table>
<thead>
<tr>
<th>Primer sequences</th>
<th>Target gene</th>
<th>PCR conditions</th>
<th>No. of cycles</th>
<th>Amplicon size (bp)</th>
<th>Source</th>
<th>Accession No.</th>
</tr>
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<td>35</td>
<td>825</td>
<td>Kim and Jeong (2001) X74726</td>
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<td>Vib 2, F: 5′-TCT AGG GGA GAC GCT GGA-3′</td>
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<tr>
<td>Vib 3, R: 5′-GCT CAC TTT CGC AAG TTG GCC-3′</td>
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<td>72°C 30 s</td>
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</tr>
<tr>
<td>Cyt 1, F: 5′-ACA AAG ACG GCC GCA AAG TTG-3′</td>
<td>Cytolysin</td>
<td>94°C 30 s</td>
<td></td>
<td>388</td>
<td>Kim and Jeong (2001) M34670</td>
<td></td>
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<td>60°C 30 s</td>
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</table>

F: forward; R: reverse.

a Primer used in a tri-primers PCR (Vib 1, Vib 2 and Vib 3R) to differentiate *V. vulnificus* 16S rRNA type A and type B.

2.4.4. PCR optimization

The specificity of the primers was tested by performing PCR on DNA extracted from strains of *V. vulnificus* ATCC 27562 biotype 1, *V. vulnificus* ATCC 33149 biotype 2, *Aeromonas hydrophila* ATCC 7966, *Arcobacter butzleri* ATCC 49616 and *Arcobacter cryaerophilus* ATCC 43157, *Escherichia coli* ATCC 25922 and from lab strains of *V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae*. To determine the analytical sensitivity of the PCR, the DNA extracted from tenfold serial dilution (10^0 to 10^-8) of a *V. vulnificus* ATCC 27562 culture was used, beginning with an optical density of 0.1 at 600 nm. Quantification of *V. vulnificus* present in each suspension, expressed as CFU ml^-1, was performed by plate counts using Bacto Marine 2216 (Difco) agar incubated at 37°C, for 24 h. Evaluation of *V. vulnificus* cells in each dilution was obtained by DAPI (4′6-diamidino-2-phenylindole) direct counts.

2.4.5. Sequence analysis

PCR products obtained by the amplification of the 16S rRNA and cytotoxin target regions from all strains identified as *V. vulnificus* and from ten randomly selected environmental samples were used for nucleotide sequencing. All specific amplicons of the expected size complementary to this region were purified by using the Wizard SV Gel and PCR clean-up System (Promega) according to the manufacturer’s instructions. The amplified fragments were directly sequenced on both strands using the same PCR primers Vib 2 and Vib 3. The sequencing was performed by Genelab c/o ENEA Casaccia Via Anguillarese 301, 00060 S. Maria Di Galeria Roma. The homology analyses of the PCR product sequences were carried out at the internet website BCM Search Launcher server (http://searchlauncher.bcm.tmc.edu/) [38]. BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) was used to conduct homology searches of the GenBank database [2] and the *V. vulnificus* YJ016 Genome Projects available at the web site (http://gib.genes.nig.ac.jp/single/main.php?spid=Vvul_YJ016).

Multiple sequence alignments were carried out using the ClustalW software (http://www.ebi.ac.uk/clustalw/) [40].

3. Results

3.1. Physico-chemical parameters

Seawater temperature varied from 14 to 23°C, salinity ranged from 37.4 to 38.2‰ (Table 2) and pH from 7.2 to 8.1.

3.2. Plankton analysis

The abundance of large plankton ranged from 178 to 616.67 individuals per m^3 of seawater and was largest in
August. Copepods accounted for 85.17% on average. Cladocerans (av. 4.77%), ostracods (av. 3.02%), chaetognates (av. 2.20%) and molluskian pteropoda (av. 1.86%) represented the main groups. The greatest abundance of small plankton was observed in April, and comprised predominantly nauplii of crustaceans and tintinnids.

3.3. Culture assays

Quantities of Vibrio spp. (mean values) ranged from 1 to 4.5 × 10 CFU l⁻¹ in large plankton, from 3.2 to 2.4 × 10² CFU l⁻¹ in small plankton and from 4.5 × 10 to 5.0 × 10³ CFU l⁻¹ in seawater samples (Fig. 1). V. vulnificus was isolated from large plankton in October 2002 and from small plankton in September 2003. In October 2003 it was isolated from seawater, and small and large plankton samples (Table 2). The highest number of V. vulnificus strains were obtained from plankton samples (24 strains were isolated from plankton and 2 from seawater). The relative percentages of V. vulnificus isolates with respect to Vibrio spp. abundance were 3.70, 21.43 and 10.53 for seawater, small and large plankton samples, respectively [16]. The strains, identified as V. vulnificus by the API 20E system, were arginine-dehydradase (ADH)-negative, lysine-decarboxylase (LDC)-positive and ornithine-decarboxylase (ODC)-negative, except for two, which were ODC+. All belonged to biotype 1 (indole-positive) strains that are known to be pathogenic for humans.

3.4. Molecular assays

The specificity of PCR assay with the primers used gave positive results only with V. vulnificus DNA; no other bacterial DNA examined gave positive results (data not shown).

PCR amplification with Cyt 1 and Cyt 2 primers of ten-fold serial dilutions of V. vulnificus DNA indicated that at least 75 pg ml⁻¹ of nucleic acid, the equivalent of 40 cells ml⁻¹, were required to yield a visible fragment on agarose gel electrophoresis. The limits of sensitivity increased 10-fold with the use of Vib 2 and Vib 3R primers and detection of V. vulnificus was observed over a range of 10²–10⁸ CFU ml⁻¹, with a lower limit of 7.5 pg ml⁻¹ of nucleic acid, or the equivalent of four cells per milliliter. All isolates identified using traditional methods were confirmed as V. vulnificus by PCR assay using both primers targeting cytolysin and 16S rRNA genes. A tri-primer PCR amplification assigned all strains to 16S rRNA type B.

In Table 2 the direct detection of V. vulnificus genes is shown using Vib 2 and Vib 3R and Cyt 1 and Cyt 2 primers in environmental samples.

Specific amplicons were not observed in March samples for either gene. Amplicons of the expected sizes, complementary to regions of 16S rRNA, were observed from April to October in large and small plankton samples, and from June to October in seawater samples. Using Cyt 1 and Cyt 2 primers the PCR assay led to detection of V. vulnificus associated with large plankton from April to October, associated with small plankton from May to October, and free-living in seawater from June to October. PCR assay targeting the 16S rRNA region produced higher numbers (38/48) of positive reactions than those obtained by the cytolysin target gene (36/48). Direct detection of V. vulnificus genes in plankton samples was more frequent than in seawater and was indifferent to low temperature values (Table 2).

When subjected to a BLASTN search, 16S rRNA sequence analysis revealed that all isolates were V. vulnificus strains. It was found that the partial 16S rDNA sequences were identical to the corresponding sequence of V. vulnificus biotype I UQM 3032 (GenBank accession no. AY 264936).

A comparison of the sequences of ten randomly selected PCR products obtained from seawater and plankton samples with known 16S rRNA gene sequences in the GenBank database showed that there was complete homology between our amplified products and the V. vulnificus I UQM 3032 sequence.

4. Discussion

V. vulnificus is a halophilic bacterium present in coastal and estuarine ecosystems throughout the world. Several studies have reported its presence along the coastal waters of the United States and in Denmark [19], Hong Kong [10], and Japan [3,15]. The abundance of V. vulnificus in a coastal environment has been linked to water temperature, while its relationship to salinity is less clear. The level of V. vulnificus is much lower during winter than during the summer months. In estuarine waters of eastern North Carolina V. vulnificus was isolated only when water temperatures were between 15 and 27°C [37]. But in the Chesapeake Bay, V. vulnificus was isolated from seawater and oysters at 8.0 and 7.6°C, respectively, using a DNA oligonucleotide probe [43] and from Danish coastal waters at 7°C, using enrichment of 250 ml water samples [19]. These studies demonstrated that very different results have been obtained using different methods. These differences could also be due to the levels of contami-
Table 2
Demonstration of *V. vulnificus* in environmental samples (SW: seawater; SP: small plankton; LP: large plankton) by PCR and culture methods; seawater temperature (°C) and salinity (%) values

<table>
<thead>
<tr>
<th>Month '02</th>
<th>T (°C)</th>
<th>S (%)</th>
<th>Method</th>
<th>SW</th>
<th>SP</th>
<th>LP</th>
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<td>38.20</td>
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<td>16S rRNA</td>
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<td></td>
<td>Cytolysin</td>
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<tr>
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nation of investigated geographic areas. The low numbers of isolates or the absence of isolation may be a consequence of the few surviving cells of *V. vulnificus* at temperatures lower than previously reported, and these forms were not enumerated by the CFU method. Presumably, very few cells are cultured at lower temperatures and it is the multipli-
cation of these few cells during the warmer months that results in their “re-emergence”. The abundance of *V. vulnificus* registered on the east coast of the USA ranged from 3.0 \times 10^{-1} to 2.1 \times 10^{2} CFU ml\(^{-1}\) [43]. Recently, Pfeffer et al. [37] reported values between 0.01 and 23.0 CFU ml\(^{-1}\) in North Carolina. A relatively low incidence of *V. vulnificus* was found in estuaries on the west coast [20] compared with the Gulf and Atlantic Ocean. Finally, in a subtropical area of the Gulf of Mexico, Lipp et al. [25] counted 0.58–1.21 \times 10^{4} CFU ml\(^{-1}\). Low densities (between 0.8 and 19 CFU l\(^{-1}\)) were detected in northern Europe [19]. Cultural assays demonstrated a lower incidence of *V. vulnificus* in the coastal environment of the Mediterranean Sea than in other investigated geographical zones, even when the number of culturable *Vibrio* spp. was similar or even higher. It allowed to suppose that saprophytic *Vibrio* species may adapt better to biotic and abiotic factors (as salinity) present in the Mediterranean Sea [5,29].

In this study *V. vulnificus* was isolated only when the water temperature ranged from 20 to 21.5°C, indicating a strong dependence of the cultivable forms with water temperature. On the contrary, salinity values here reported (37.4–38.2‰) were consistently higher than the maximum requirements for optimal growth of *V. vulnificus* [21]. Most strains were phenotypically similar to *V. vulnificus* ATCC 27562 biotype 1, except for four strains unable to grow at NaCl 8%. All isolates were assigned to 16S rRNA type B as reported for the majority of environmental strains isolated from the Korean coast [23] and appear to be the dominant strains.

Positive PCR reactions on total DNA without cultivation obtained using the 16S rRNA target region were higher than those obtained using the cytolysin target gene. The latter is a structural region correlated with the virulence of the cell and possesses strong significance in epidemiological studies. Nevertheless, this technique suffered from a loss of sensitivity which was also measured in our experiment. One reason for the greater number of PCR-positives retrieved by the 16S rRNA target primers could be the higher number of ribosomal operons in comparison with cytolysin gene copies in the *V. vulnificus* genome. The PCR survey conducted over a 2-year period showed that in March *V. vulnificus* genes were undetectable. In April and May they were detected in plankton samples.

The occurrence of *V. vulnificus* as plankton-associated confirms the role of plankton as its potential reservoir [16,39]. Present data are consistent with our previous findings on the association of pathogenic bacteria with planktonic organisms in the Mediterranean environment [9,14,29].

The detection of *V. vulnificus* by molecular assay was possible even when we did not manage to isolate it. This can be attributed to the fact that *V. vulnificus* was in a viable but non-culturable (VBNC) state due to low nutrient and low-temperature environments [34]. A recent review [17] of the methods used so far to culture and detect *V. vulnificus* stressed the importance of designing an appropriate detection strategy, especially when the target species was present in low numbers. Detection by PCR assays appears highly specific and relatively less time-consuming than conventional methods.

Rapid and dependable identification of *V. vulnificus* in marine samples is essential in order to reduce the potentially hazardous effects of widespread environmental contamination.

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**References**


