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Occurrence and expression of virulence-related properties of *Vibrio* species isolated from widely consumed seafood products

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Abstract

In this study, widely consumed fresh seafood products were examined for the presence of *Vibrio* spp. Thirteen percent of the samples examined were found to be contaminated with halophilic vibrios belonging to the species *V. alginolyticus* (81.48%), *V. parahaemolyticus* (14.8%) and *V. cholerae* non 0:1 (3.7%). A greater isolation frequency (18.9%) was found for mussels. Significant adhesiveness and strong cytotoxicity factors were revealed in a significant number of the *Vibrio* spp. isolated. These results confirm that the presence of *Vibrio* spp. in seafood products is common, and suggest that routine examination of such products for these pathogenic agents would be advisable.

Keywords: *Vibrio* spp.; Seafood; Virulence; Properties

1. Introduction

Several recent outbreaks of cholera in Italy and other areas of Europe (OMS, 1994) have fostered a renewed interest in species of the genus *Vibrio* among researchers in these countries. Particular attention is now being focused on *V. parahaemolyticus*, which has often been held responsible for food poisoning (Honda et al., 1992), and on *V. cholerae* non 0:1, which has been found to be involved in cholera-like infections of the intestinal tract and other systems (Piersimoni et al., 1991; Abbott et al., 1992).

However, in recent years we have witnessed an increasing awareness of the importance of other halophilic *Vibrio* spp., including *V. vulnificus*, *V. mimicus*, *V. fluvialis*, *V. hollisae*, *V. damsela* and *V. alginolyticus*. Although these species make up part of the normal autochthonous flora in water, they are often involved in both gastrointestinal illness and septicemia (Blake et al., 1980; Aggarwal et al., 1986; Bockemuhl et al., 1986; Bravo Farinas et al., 1992; Jackson et al., 1997; Mouzin et al., 1997). The occurrence of gastrointestinal illness is linked to various virulence factors, including adhesiveness and invasiveness, and lesional factors such as exoenzymes and enterotoxins, including those with

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potent haemolytic activity (Alam et al., 1996; Osawa et al., 1996; Chakraborty et al., 1997; Rahman et al., 1997).

Seafood products harvested from contaminated waters or which have been improperly preserved after harvesting are known to play an important role in infections by these *Vibrio* spp. (Cioglia et al., 1982; Gelosa, 1984; Mouzin et al., 1997). Accordingly, in the present study various species of fish and clams, harvested from the waters of the Adriatic Sea coast of the central Italian Region of the Marches, as well as naturally growing mussels from the same area, were subjected to bacteriological examination aimed at verifying the presence of various *Vibrio* spp. (with particular reference to halophilic species) in these food products and determining virulence markers for the selected isolates.

The ultimate goal of this research was to gain further insight into the epidemiological problems related to the genus *Vibrio* and its involvement in gastrointestinal infections.

2. Materials and methods

2.1. Sites and sampling

From the winter of 1996 through the autumn of 1997, 200 samples of the most widely consumed seafood products in our Region were collected by trawling and dredging for shellfish in the coastal waters of the area of the Adriatic Sea between the town of Falconara Marittima and the Conero River (Fig. 1). Among the samples collected, 114 were fish, 37 were mussels and 49 clams. The fish examined included: anchovies (*Engraulis sardina*),

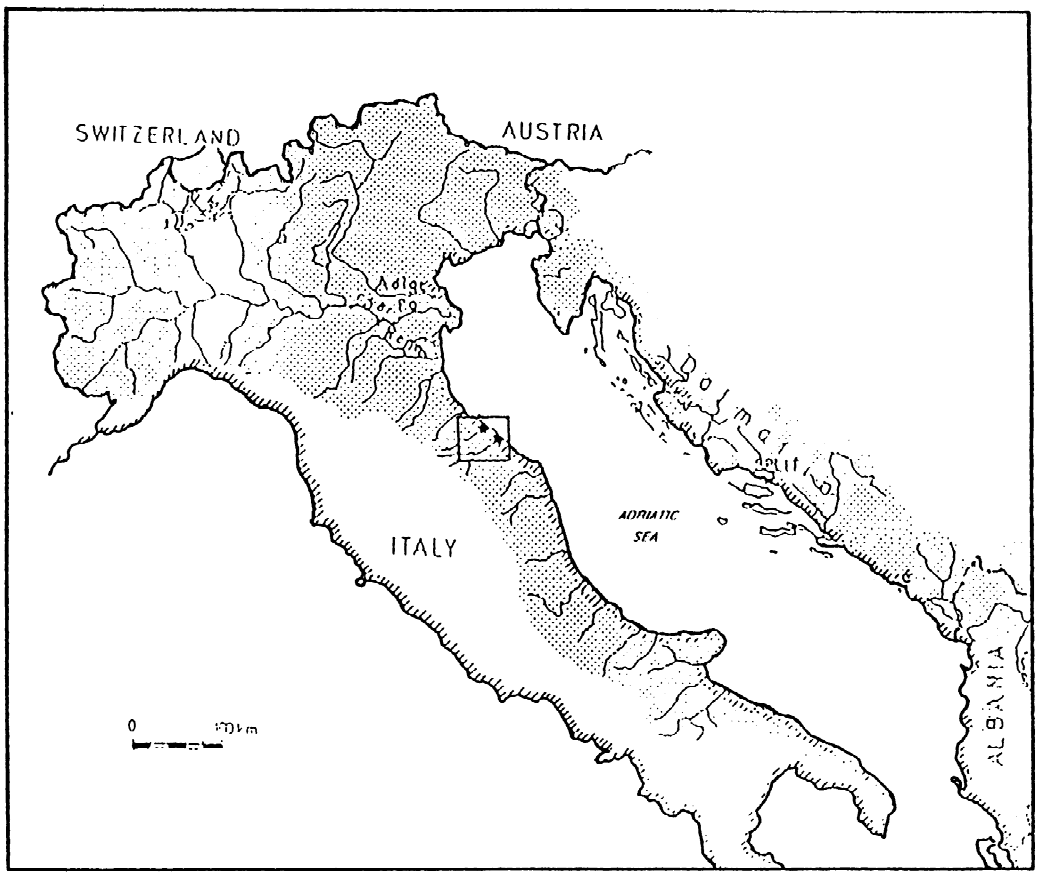

Fig. 1. Map of the area from which the samples described in this study were obtained.

gray mullet (*Mugil cephalus*), sardines (*Sardina sardina*), mackerel (*Scomber scomber*), red mullet (*Mullus surmuletus*) and other species common to the Adriatic Sea. The edible Lamellibranchiae examined were mussels (*Mytilus galloprovincialis*) and clams (*Venus gallina).*

2.2. Bacteriological analysis

Each sample consisted of five (in the case of the larger species) or 10 specimens (in the case of the small- to medium-sized fish and the molluscs).

The samples were prepared as follows: (i) the fish were examined by sampling the gills and visceral organs under aseptic conditions. Tests for *Vibrio* spp. were carried out on homogenates of this material appropriately diluted in four volumes of H_2O at 3% NaCl (w/v); (ii) the molluscs were removed from their shells and homogenised along with their intravalvular water, after the addition of four volumes of H_2O at 3% NaCl (w/v).

In both cases, testing for *Vibrio* spp. was carried out on the homogenates using the following steps.

2.2.1. Enrichment and isolation

Ten millilitres of homogenate was mixed with 40 ml of alkaline peptone H_2O (pH 8.6) at 3% NaCl (w/v) and incubated at 37°C for 24 h. Isolation on TCBS (Oxoid, Garbagnate M. se, Milano, Italy) plates was carried out with incubation at 37°C for 24 h.

2.2.2. Identification

Suspected colonies, i.e. those observed to be yellow and/or green in colour, were identified as follows: gram staining; production of cytochrome oxidase and catalase; fermentation activity in Triple Sugar Iron (TSI) (Oxoid); resistance to vibriostatic agent 0/129 (plates at concentrations of 10 and 150 μg) (Oxoid); growth in peptone water at different NaCl concentrations (0, 3, 6, 8 and 10%); reduction of nitrates to nitrites. These tests were performed as described in *Methods for General and Molecular Bacteriology* and *Bergey's Manual* (Gerhardt et al., 1994; Holt et al., 1994). A more complete genus identification was obtained using the API 20E system (Bio-Merieux).

For species identification, an innovative method proposed by Alsina and Blanch (1994) was employed. Briefly, starting from the combination of the arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase tests (A/L/O), it is possible to proceed to six blocks or 'keys' of biochemical reactions, for a total of 38 tests.

Finally, with regards to the differentiation between the agglutinable and nonagglutinable biotypes of *V. cholerae*, serological typing was performed using 0:1 polyvalent serum (Oxoid).

2.3. Pathogenic properties

The *Vibrio* strains were also assayed to evaluate several virulence markers, e.g. urease and haemolytic activity (production of thermostable direct haemolysin, TDH), adhesion to cell lines and ability to secrete toxins.

2.3.1. Adhesiveness and cytotoxicity tests

2.3.1.1. Bacterial preparations

Vibrio spp. were inoculated in brain heart infusion broth (BHI) (Oxoid) supplemented with 0.5% NaCl and grown in 25 ml flasks incubated at 37°C for 18 h with agitation at 150 rpm. For the supernatant cytotoxicity assay, cell-free filtrates were prepared by centrifugation (3000 rpm) at 4°C for 30 min, with subsequent filtration of the supernatants through a 0.45 μm pore size filter (Millipore, Vimodrone, Milano, Italy). The filtrates were either refrigerated prior to immediate use or stored at −80°C. For both the test of adhesiveness and the bacterial cytotoxicity assay, exponentially growing bacteria were washed three times in PBS and resuspended in serum-free Dulbecco's Modified Eagle Medium (D-MEM) (Sigma, Milano, Italy).

2.3.1.2. Cell culture preparation

Hep-2 and CHO cells were grown in D-MEM containing 10% foetal calf serum (FCS) (Flow Laboratories, Irvine, UK), 1% nonessential amino acids, 5 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded (5×10^4 cells/ml) on 13 mm diameter glass coverslips in separate wells or in 96-well tissue culture plates at

37°C for 24 h in an incubator containing an atmosphere of 95% air in 5% CO_2.

2.3.1.3. Assay for bacterial adhesiveness and cytotoxicity

Bacteria prepared as described above were added to Hep-2 cells at a multiplicity of infection (MOI) of 100 in serum-free D-MEM before incubation for 90 min at 37°C.

After this step, cells were washed three times in serum-free D-MEM to remove nonadherent bacteria, fixed in methanol and stained with May Grewnvald-Giemsa. Bacterial adhesiveness and the cytotoxicity due to bacteria were assessed microscopically. Adhesive capacity was expressed as the percentage of cells with more than 10 bacteria per cell and bacteria were considered able to induce cytotoxicity when $\geq$50% of cells became rounded.

For supernatant cytotoxicity assay the CHO monolayers were maintained in D-MEM containing 10% FCS. Serial double dilutions of filtrates starting from 1:2 were tested for 24 h. Isolates showing a cytotoxic effect (i.e. 50% of rounded cells) at a dilution of 1:10 or more were regarded as positive.

For fluorescence microscopy, CHO cells were fixed with 3.7% formaldehyde in PBS with 2% bovine serum albumin for 10 min at room temperature. After washing in the same buffer, cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 10 min at room temperature. For F-actin detection, cells were stained with fluorescein-phalloidin (FITC-phalloidin) (Sigma, working dilution 1:500) at 37°C for 30 min. After washing, coverslips were mounted with glycerol-PBS (2:1) and analysed with an Olympus fluorescence microscope.

2.3.2. Production of thermostable direct haemolysin

In order to observe the Kanagawa phenomenon, Wagatsuma agar (Takeda, 1983; Elliot et al., 1992; Atlas, 1993) containing rabbit erythrocytes was inoculated with 0.001 ml of a 0.5 ml standard McFarland suspension of each *Vibrio* strain to be tested. The plates were incubated overnight at 35°C and positive reactions were recorded as a zone of β haemolysis that develops around the spot of growth on the rabbit blood plate.

2.3.3. Urease activity

Urease activity was determined on medium containing Christensen urea at 1% NaCl plus 5% urea, cooled in a slanted position. The test tubes were incubated for 24 h at 37°C. Hydrolysis of the urea was revealed by the appearance of a red colour, which is an index of alkalinization of the entire medium.

COD.18 *V. alginolyticus* and COD.66 *V. parahaemolyticus* (kindly supplied by Dr. Donatella Ottaviani of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Ancona, Italy) were included as reference strains.

3. Results

3.1. Presence of Vibrio spp. in seafood products

As shown in Table 1, 26 of the fresh seafood samples examined (13.0%) contained *Vibrio* spp. The highest percentage of contamination was found in mussels (18.9%).

The most frequently isolated species was *V. alginolyticus* (in 24 of 30, or 80.0% of the samples tested), whereas five strains of *V. parahaemolyticus* were found (16.7%), three in fish, one in clams and one in mussels. The simultaneous presence of these two strains was found in one clam sample and two fish samples; moreover, the simultaneous presence of *V. alginolyticus* and *V. cholerae* non 0:1 was found in only one sample.

Table 1
Isolation frequency of *Vibrio* spp. in fresh raw seafood products

Sample type	No. of samples examined	Samples positive for *Vibrio* spp.		No.	Species isolated
		No.	%		
Fish	114	15	13.15	14	*V. alginolyticus*
				3	*V. parahaemolyticus*
Mussels	37	7	18.90	6	*V. alginolyticus*
				1	*V. parahaemolyticus*
Clams	49	4	8.16	4	*V. alginolyticus*
				1	*V. parahaemolyticus*
				1	*V. cholerae* non 0:1
Total	200	26	13.0	30	

3.2. Pathogenic properties of Vibrio spp.

3.2.1. Bacterial adhesion and cytotoxicity

The *Vibrio* strains were assayed for their adhesiveness and cytotoxicity in a Hep-2 cell line. Approximately 50% of the strains was found to adhere to the cells, but adhesion of bacteria to all (100%) of the cells was observed in only 10% of the samples (two strains of *V. alginolyticus* and one of *V. parahaemolyticus*) (Table 2, Fig. 2).

A significant number of strains of the *Vibrio* spp. was able to produce cytotoxic effects, although with a varying degree of intensity depending on the species. In fact, all of the *V. parahaemolyticus* strains were strong producers of cytotoxins as was the one strain of *V. cholerae* non 0:1, but only three strains of *V. alginolyticus*, as shown in Table 2 and Fig. 2.

3.2.2. Supernatant cytotoxicity

The ability of the strains to produce and secrete into the supernatant cytotoxic factors able to produce a cytopathic effect (CPE) in Chinese hamster ovary (CHO) cells in culture was also tested.

Only the one strain of *V. cholerae* non 0:1 isolated

Table 2
Distribution of virulence properties among *Vibrio* spp. strains isolated from widely used seafood products

Species	Adhesiveness (% of cells with >10 bacteria on the surface)	Bacterial cytotoxicity[a]	Supernatant cytotoxicity[b]
V. alginolyticus			
	>80		+ + −
	<50	−	−
	>50	+	−
	>80	−	−
	>90	+ +	−
	>90	+	−
	<20	−	−
	<20	−	−
	>80	−	−
	<10	+ +	−
	<20	+	−
	>10	−	−
	100	+ + + +	−
	>10	−	−
	>90	+ + + +	−
	−	−	−
	>80	−	−
	100	+	−
	<50	+ + +	−
	−	−	−
	−	+	−
	>10	−	−
	>80	−	−
	>90	+ + + +	−
V. parahaemolyticus			
	>50	+ + + +	−
	>80	+ + + +	−
	>80	+ + + +	−
	100	+ + + +	−
	<20	+ + + +	−
V. cholerae non 0:1			
	>90	+ + + +	+

[a] + + + +, High degree of cytotoxic intensity; + + +, medium degree of cytotoxic intensity; + +, low degree of cytotoxic intensity.

[b] +, Positive; −, negative.

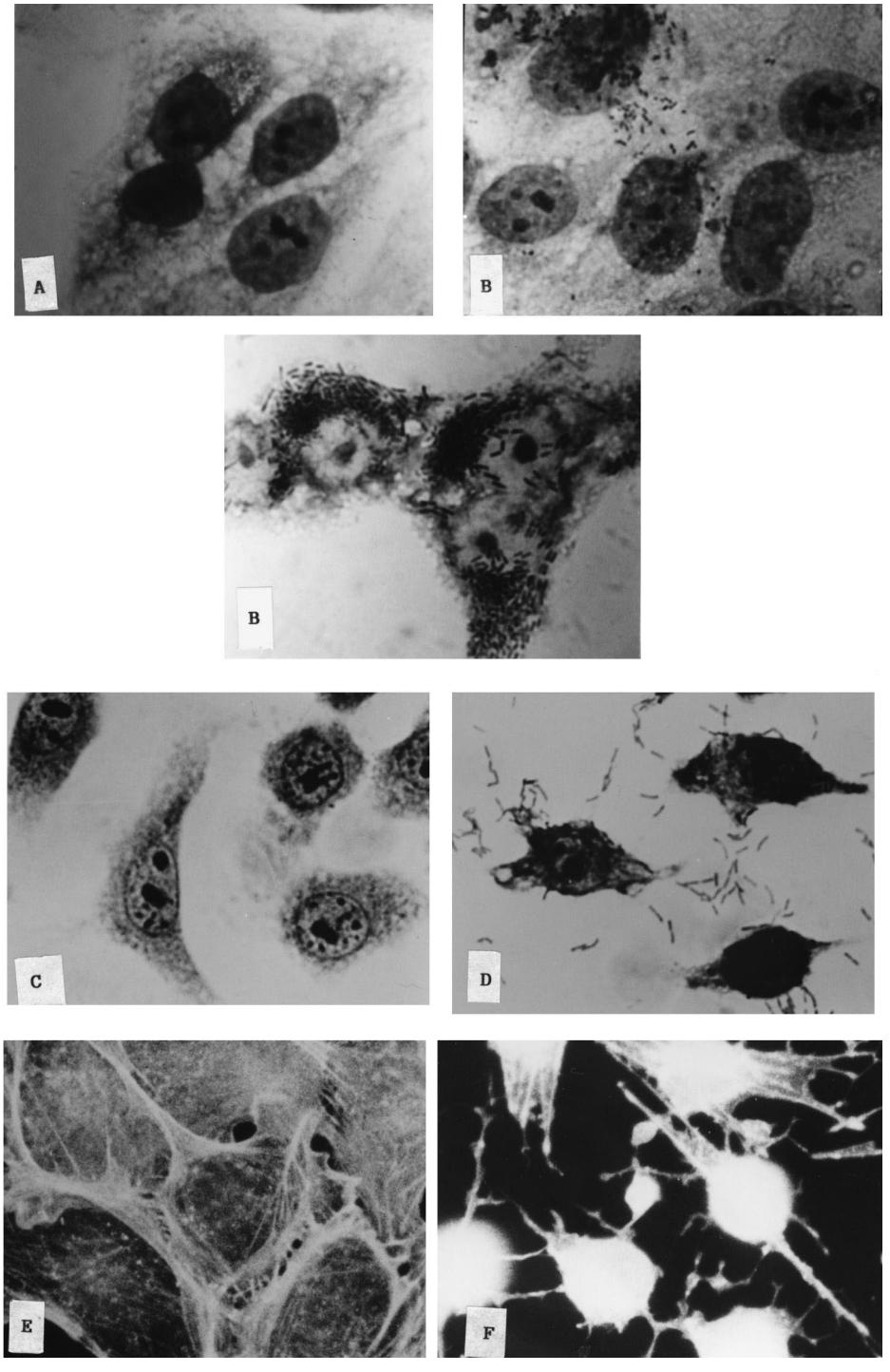

Fig. 2. Adhesiveness and cytotoxic activity of *Vibrio* spp. isolated from fresh raw seafood. Adhesion of isolates to Hep-2 cells (B) as compared to control (A). Phase contrast micrographs of the cytotoxic-like effect of bacteria on Hep-2 (D) as demonstrated by the rounding of these cells as compared to controls (C). Fluorescence micrographs reveal a disruption of the F-actin network in CHO cells (F) by staining with fluorescein-phalloidin. (E) normal CHO cells.

Table 3
Distribution of some virulence factors among *V. parahaemolyticus* strains related to the production of hemolysin and urease activity

Adhesiveness (% of cells with >10 bacteria on the surface)	Bacterial cytotoxicity[a]	Supernatant cytotoxicity[a]	Urease[b]	Hemolysin[b]
>50	+ + + +	–	+	−
>80	+ + + +	–	+	−
>80	+ + + +	–	−	+
100	+ + + +	–	−	−
<20	+ + + +	–	−	−

[a] + + + +, High degree of cytotoxic intensity.
[b] +, Positive; −, negative.

was found to be able to induce CPE, causing shrinkage and rounding of the cells. Since such changes in cell morphology are known to be dependent on cytoskeletal actin, we investigated the cellular organisation of F-actin by staining the cells with fluorescein-phalloidin. Fluorescence micrographs (Fig. 2) reveal a disruption of the F-actin network in treated cells.

3.2.3. Haemolysin and urease activity

Haemolysin and urease activities were assayed only for the strains of *V. parahaemolyticus*. One strain tested positive for the Kanagawa phenomenon, but negative for the ability to hydrolyse urea, while positive urea test results were obtained for two strains. No strain was found to be simultaneously positive for the two activities (Table 3).

4. Discussion

In the present study aimed at evaluating the presence of halophilic *Vibrio* spp. in fresh seafood products, the presence of these agents was in fact revealed in 13.15% of the samples examined. This value is lower than those reported by other Italian authors, who reported isolation frequencies ranging from 34 to 84% (Boccia et al., 1978; Schintu et al., 1994; Simioli et al., 1994; Ottaviani et al., 1995).

The highest isolation frequencies were found in the mussel samples, due both to their peculiar innate characteristic of filtering large amounts of water and to the fact that, given the free-growing nature of the specimens examined, they had grown in uncontrolled waters subject to contamination.

In agreement with data reported in the literature (Huq et al., 1983), our study also found a seasonal isolation trend characterised by a greater presence of *Vibrio* spp. in the warmer months of the year.

The most frequently isolated species was *V. alginolyticus*, in agreement with results obtained by other Italian authors and those elsewhere (Schintu et al., 1994; Simioli et al., 1994; Ottaviani et al., 1995), who reported a greater diffusion of this species in mussels.

Of particular interest was the finding of a strain of *V. cholerae* non 0:1, a species which, in Italy, has repeatedly been found to have been involved in cases of gastro-enteritis and in other severe pathologies affecting various systems, given its invasive nature and its ability to provoke bacteremia (Lessner et al., 1985; Janda et al., 1986; Xercavins et al., 1989; Hansen et al., 1990).

The block biochemical classification method (Alsina and Blanch, 1994) used in this study allowed a clear identification of all the strains isolated. The small variety of species found, which was limited to *V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae* non 0:1, confirms the results of other studies carried out on samples taken from the same coastal area (Ottaviani et al., 1995). This finding may be explained by the fact that all of the samples examined were taken from a small area of the coastline of the Marches, and therefore from an homogeneous microbiological habitat. On the contrary, the use of Alsina's set of biochemical keys by other researchers in the study of seafood products from elsewhere (Ottaviani et al., 1995) revealed a great variability in the species isolated, indicating that the routine application of this method would be worthwhile, in particular in the field of food microbiology.

Although halophilic vibrios are known to produce

a series of virulence factors, many questions remain to be answered with regard to the mechanisms of their pathogenicity. In fact, most of the virulence factors identified in the different species are not directly associated with pathogenicity, which would thus appear to be strain-specific and due, in particular, to the production of cytotoxic substances (Spira and Fedorka-Cray, 1984).

V. alginolyticus, which is widespread in the marine environment, does not appear to have a strong pathogenic effect. In fact, it was shown to have only slight effects in vivo on new-born mice (Kawagishi et al., 1995) and is rarely associated with pathological phenomena in humans. With regards to *V. alginolyticus*, our results are in agreement with what has been reported in the literature, and confirm that the pathogenicity of this species is strain-specific (Ottaviani et al., 1995).

As regards *V. parahaemolyticus*, Kanagawa haemolysin or thermostable direct haemolysin (TDH) is conventionally considered to be the main virulence factor for this species (Joseph et al., 1982; Osawa et al., 1996), and is characteristic above all of strains of clinical origin (Takeda, 1983). In the present study, a single strain of *V. parahaemolyticus* (20%) was found to produce TDH. Our results therefore confirm the findings of other authors who found a lower percentage of Kanagawa-positive strains among environmental isolates with respect to those from clinical samples (Sakazaki et al., 1968; Kelly and Stroh, 1989). Among the biochemical characteristics of *V. parahaemolyticus*, urease activity (Uh^+) has usually been considered to be associated with Kanagawa-positive (K^+) strains, and therefore to be predictive for the production of haemolysin (Nishibuchi et al., 1992; Kaysner et al., 1994). Other authors have, on the contrary, found both phenotypes Uh^+ and K^- and strains Uh^- and K^+ isolated from patients with gastro-enteritis in various part of the world (Kelly and Stroh, 1989). Therefore, our results reporting the isolation of both K^+ and Uh^- strains as well as K^- and Uh^+ strains lead us to assume that the urease test is not predictive for TDH production, and that haemolysin is therefore not a unique characteristic for the pathogenesis of *V. parahaemolyticus* infections.

With regard to the other virulence factors considered, significant adhesiveness and a strong cytotoxic effect were found for all of the strains of *V. parahaemolyticus*, thus allowing us to attribute an important role for this species in the development of gastro-enteritis in humans through seafood consumption.

With regard to *V. cholerae* non 0:1, which is a notorious cause of cholera-like syndromes of varying severity, different virulence factors would appear to be involved (Singh et al., 1996a,b). These are for the most part toxic factors such as thermostable toxin, Shiga-like toxin, etc. In our work, positive results were found for all of the markers assayed for a single strain of *V. cholerae* non 0:1. Similar results (data not shown) were also obtained for the strains of *V. cholera* non 0:1 isolated from brackish water, from which the sample of clams from which the strain of *V. cholera* non 0:1 was also taken. Therefore, these data confirm the importance of this species as a cause of infections in humans as a result of the consumption of crude or under-cooked seafood products and of contaminated seawater.

The results of our study also indicate that, even if in low percentages, potentially pathogenic *Vibrio* spp. can be present in seafood products and that, as a consequence, these foods can play a significant role in the transmission of these micro-organisms to humans.

We therefore agree with the proposal of other authors (Urdaci et al., 1988; Schintu et al., 1994; Simioli et al., 1994) that, alongside routine testing for faecal contamination, testing for micro-organisms such as halophilic *Vibrio* spp. should be carried out on these food products. Moreover, an efficient monitoring strategy should include health education programs aimed at eliminating the errors and omissions which are often at the root of toxin infections. These include the cooling of freshly caught seafood products with contaminated seawater, the presence of illicit wastewater elimination in waters destined for the farming of molluscs and, lastly, the habit on the part of the human population of harvesting molluscs in uncontrolled areas.

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