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Prevalence and virulence properties of non-O1 non-O139 *Vibrio cholerae* strains from seafood and clinical samples collected in Italy

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ABSTRACT

Seafood and clinical samples collected in Italy during 2006 were analyzed to evaluate prevalence, serological and virulence properties of non-O1 non-O139 *Vibrio cholerae* (NCV) isolates. Biochemical and serological characterization of the strains was performed by standardized procedures while virulence properties of NCVs were assayed by molecular, *in vivo* and *in vitro* toxicological methods. Of the 300 seafood samples examined, including mussel, cod, mackerel, anchovy, clam, prawn and cuttle-fish, 5.6% were positive for NCVs: 4.7% and 8.5% from local and imported seafood, respectively. The prevalence of NCVs was highest in prawn (16.6%) and mussel (7.7%). Of 58 hospitalized patients that presented acute diarrhea, 3.4% eliminated NCVs in stools 24–48 h after consumption of seafood. All NCVs had *ToxR* and *hlyA*ET genes but lacked *ctxA*, *zot*, and *tcpA* genes. One isolate from prawn had *stn*/*sto* gene. All strains were hemolytic, cytotoxic, and able to induce intestinal and extraintestinal effects on the suckling mouse model. Our results confirm that non-toxigenic NCVs that express the gene encoding El Tor-like hemolysin can be isolated from patients suffering a cholera-like syndrome after consumption of seafood. This evidence along with the virulence and enteropathogenicity of all the *ctxA*$^-$ *tcpA*$^-$ *zot*$^-$ *stn*/*sto*$^-$ *hlyA*ET$^+$ NCV isolates in the experimental model, suggest that El Tor-like hemolysin may play an important role in human pathogenesis. Moreover, the isolates from seafood showed molecular, biological and enzymatic patterns similar to those isolated from clinical samples, underlining that environmental NCVs are potentially able to induce human infections and confirming the important role of seafood as a vehicle of *V. cholerae* diseases.

1. Introduction

Vibrio cholerae can be divided into 2 major groups: cholera-causing strains of the serogroups O1 and O139, and non-O1 non-O139 cholera vibrios (NCVs) (Blake et al., 1980; Sack et al., 2004). NCVs exist as part of the normal bacterial flora of estuarine and coastal waters and they have been considered of negligible microbiological significance for a long time. However, in the last decades it has been demonstrated that NCVs can cause sporadic cases or occasional outbreaks of diarrhea in humans (Blake et al., 1980; Faruque et al., 2004), acute septicemia (Namdari et al., 2000; Restrepo et al., 2006) and skin infections (Blake et al., 1980) through the ingestion of seafood (CDC, 1982; Namdari et al., 2000; Crump et al., 2003) or exposure to aquatic environment (Lukinmaa et al., 2006). Individuals with liver disease or an immunosuppressive condition are more vulnerable to NCV extra-intestinal infections (Restrepo et al., 2006). The gastrointestinal infections caused by NCVs usually have a favourable outcome (Namdari et al., 2000), while invasive infections may be fatal (Lukinmaa et al., 2006). A wide range of putative virulence factors has been associated with NCVs. These include cholera toxin (CT) (Jiang et al., 2003), toxin-coregulated pilus (TCP) (Sarkar et al., 2002), ToxR regulon that permits the expression of CTX and TCP (Waldor and Mekalanos, 1994), heat stable enterotoxin (NAG-ST) (Ogawa et al., 1990; Guglielmetti et al., 1994), zonula occludens toxin (ZOT) (Jiang et al., 2003), El Tor-like hemolysin (Ichinose et al., 1987; Iyer et al., 2000; Zhang and Austin, 2005), cytotoxic factors (Namdari et al., 2000; Figueroa-Arredondo et al., 2001; Faruque et al., 2004; Restrepo et al., 2006; Saka et al., 2008), and exocellular enzymes (Iyer et al., 2000). However, the exact mechanism of NCV pathogenesis remains unclear. For this reason, the study of the virulence factors in clinical as well as environmental strains of NCV around the world would be of great value to elucidate the pathogenesis of non-toxigenic *V. cholerae* disease. To increase the epidemiological importance of these micro-organisms, is the evidence that NCVs are involved in the genesis of

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Fig. 1. Geographical locations of sampling sites.

newer variants of *V. cholerae* by horizontal gene transfer from O1 to non-O1 serogroups (Bik et al., 1995). In addition, the possible conversion of non-O1 to O1 serotype has provided added interest (Colwell et al., 1995). Moreover, the transfer of *ctxA* and *tcpA* genes from toxigenic *V. cholerae* O1 to environmental NCVs has been demonstrated (Faruque et al., 1998b; Karaolis et al., 1999). Previous studies have shown that NCVs are more capable of survival and multiplication in a wide range of seafoods than *V. cholerae* O1, O139 (Roberts and Gilbert, 1979; DePaola et al., 1987) and this ability implies a potential extension in the range of possible sources for human infection. During past decades in Italy, small outbreaks associated with eating raw or undercooked shellfish have been documented (CDC, 1981; Piergentili et al., 1984). Despite this, few studies, from limited geographical areas, on the presence of NCVs in mussels (Ripabelli et al., 1999), and no surveillance studies for toxigenic and non-toxigenic *V. cholerae* diseases have been conducted in Italy. The present report describes, for the first time in Italy, an extensive investigation to evaluate the prevalence of NCVs in local and imported seafood purchased in Italy during 2006 and in the stools of diarrheal patients collected during the same period in an hospital chosen as a sample. The second aim of our study was to determine the presence of genes encoding the most important virulence factors in NCVs from clinical and food sources, and to compare their potential pathogenicity by toxicological *in vivo* and *in vitro* assays.

2. Materials and methods

2.1. Seafood samples

During 2006, a total of 300 seafood samples were collected by the Official Veterinary Authority. Fresh seafoods from May to September were sampled from multiple sites (n) of Italian coastal waters authorized for fishing and the harvesting of mussels. The localities were categorized into three different geographical areas: Ligurian coast ($n=5$), Adriatic coast ($n=8$) and Tyrrhenian coast ($n=5$) (Fig. 1). Overall, the frequency of sampling on each site was monthly. The local seafoods sampled were mussel, cod, mackerel, anchovy, and clam (Table 1). While mussels were harvested at each sampling from all sites, the frequency of sampling of the other seafood species differed during the project period in relation to availability (Table 1).

Frozen seafood from South East Asia was sampled from January to December on arrival at the port of Ancona. The seafoods sampled were prawn and cuttle-fish imported from Japan and Thailand, respectively. The frequency of sampling differed during the project period in relation to importation activity. Fresh and frozen seafood was sampled before commercialization and transported at room temperature to the Italian Reference Laboratory for Bacteriological Contamination of Shellfish (CEREM). Transportation time ranged, as a rule, from 4 h to 6 h.

2.2. Clinical samples

From January to December 2006, a passive surveillance study for toxigenic and non-toxigenic *V. cholerae* diseases was conducted at Santa Maria Goretti Hospital of Latina (Central Italy). This city was chosen because its inhabitants frequently eat seafood. Fifty-eight stool specimens, from hospitalized patients with acute diarrhea, were examined. All samples were collected on the patients' admission to the hospital, before antibiotic treatment. The microbiological laboratory of the hospital researched for other enteric non-*Vibrio* bacteria, viruses and parasites.

Part of each sample was placed in Cary-Blair medium (Oxoid Ltd, Basingstoke, England) and transported, at room temperature, to the CEREM. Transportation time ranged, as a rule, from 4 h to 6 h. Samples collected at night or during the weekend were kept at room temperature and transported to CEREM the following day.

2.3. Culture procedures

All stool specimens and seafood samples were examined at CEREM immediately upon arrived to detect *V. cholerae*. The protocol described by the International Standards Organization (UNI EN ISO 6887-3, 2003) was followed for preparation of seafood samples. Clinical and seafood samples were analyzed to detect *V. cholerae* according to standard procedures (Farmer et al., 1985; Kaysner and DePaola, 2004).

Table 1
Distribution of NCVs in fresh seafood samples collected from various Italian locations.

	Ligurian coast					Adriatic coast					Tyrrhenian coast				
	No. (positive)					No. (positive)					No. (positive)				
	May	Jun	Jul	Aug	Sep	May	Jun	Jul	Aug	Sep	May	Jun	Jul	Aug	Sep
Mussel	5(0)	5(0)	5(0)	5(1)	5(1)	8(0)	8(0)	8(1)	8(2)	8(1)	5(0)	5(0)	5(0)	5(1)	5(0)
Clam	1(0)	2(0)	2(0)	4(0)	3(0)	3(0)	2(0)	5(0)	7(0)	7(0)	3(0)	3(0)	4(0)	5(0)	5(0)
Anchovy	1(0)	1(0)	2(0)	2(0)	2(0)	1(0)	2(0)	4(0)	4(1)	3(0)	1(0)	1(0)	1(0)	2(0)	3(0)
Cod	1(0)	1(0)	1(0)	2(0)	3(0)	1(0)	1(0)	2(0)	4(1)	4(0)	1(0)	1(0)	2(0)	3(0)	3(1)
Mackerel	1(0)	1(0)	1(0)	2(0)	1(0)	1(0)	2(0)	2(0)	2(0)	3(0)	1(0)	1(0)	1(0)	2(1)	3(0)