SHORT COMMUNICATION

Serological and molecular characterization of *Vibrio parahaemolyticus* marine strains carrying pandemic genetic markers

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In 2005, pandemic *Vibrio parahaemolyticus* was reported to have been introduced in Europe: O3:K6 strains were isolated from clinical cases in France and Spain, and were found to be associated with consumption of contaminated seafood. On the contrary, pandemic strains were not isolated from seafood or from the environment itself. Analysis of two *V. parahaemolyticus* strains isolated in May 2007 from Northern Italy seawater and plankton samples revealed the presence of the virulence gene *tdh* and the pandemic-specific markers *orf8* and *toxRS/new* sequence (group-specific PCR). The two strains showed serotypes not included in the 'pandemic group', but their molecular typing proved that they represent a single clone showing a genetic profile very similar to that of pandemic O3:K6 reference isolates. Moreover, the two marine strains carried three virulence-related genes associated with clinical strains and, to date, hardly ever or never detected in environmental strains. The presence, in strains isolated from the marine environment, of genetic pandemic markers and virulence genes normally associated with clinical isolates proves that marine strains might constitute a public health concern.

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Vibrio parahaemolyticus is a natural inhabitant of marine and estuarine environments, which causes seafood-borne gastroenteritis and rarely wound infections and septicemia. *V. parahaemolyticus*-mediated gastroenteritis has traditionally been associated with two virulence factors, thermostable direct hemolysin (TDH) and TDH-related hemolysin, which are present in most clinical isolates and have been detected with low frequency in environmental strains (DePaola *et al.*, 2003; Dileep *et al.*, 2003). The relationship between strains isolated from the aquatic environment, considered the source of the infection, and strains isolated from clinical cases is poorly understood.

Not all *V. parahaemolyticus* strains have the same pathogenic potential and, usually, infections in humans are caused by diverse serotypes. To date, the so-called 'pandemic group' includes 21 serotypes, based on nearly identical genotypes and molecular profiles, referred to as serovariants of

O3:K6 isolates (Nair *et al.*, 2007). Currently, there is no unanimity in the scientific community with regard to identifying a single marker for pandemic strains and suspected *V. parahaemolyticus* pandemic isolates have been identified by positive group-specific PCR, based on a sequence variation in the *toxRS* gene (Okura *et al.*, 2003; Han *et al.*, 2008) and the presence of the *tdh* gene.

Quilici *et al.* (2005) and Martinez-Urtaza *et al.* (2005) reported evidence that pandemic *V. parahaemolyticus* had migrated into Europe and has been isolated from clinical cases in France (Quilici *et al.*, 2005), in Spain (Martinez-Urtaza *et al.*, 2005) and in Russia during gastroenteritis outbreaks (Smolikova *et al.*, 2001).

Although in all the outbreaks reported in Europe the clinical cases have been related to the consumption of uncooked seafood or contact with contaminated seawater, this correlation has been established only on the basis of epidemiological data in that the pandemic strains were detected only in clinical samples and not in seafood or in the environment itself.

In this study, we have performed a characterization of the first European *V. parahaemolyticus* strains carrying the pandemic markers that have been isolated directly from the environment and specifically deriving from Italian North Adriatic

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water and plankton samples. The strains are here called environmental, in that they have been directly isolated from environmental samples, water, plankton and marine sediments, and to differentiate them from strains isolated from clinical cases.

To investigate the presence of pandemic strains in marine samples from the Adriatic Sea during the years 2006 and 2007, a screening was conducted (Caburlotto *et al.*, 2008) with primers *orf8* (Myers *et al.*, 2003), *tdh451* and GS-V (corresponding to the *toxRS/new* gene; Okura *et al.*, 2003) on the *V. parahaemolyticus* isolates, revealing the presence of the three genes in one of the analyzed strains (Figures 1a–c); this strain (strain VP_eVE_{pan}) was isolated in May 2007 from a marine water sample taken in a coastal site in the locality Caleri, close to the estuaries of the Adige and Brenta rivers, 500 m from the Venetian coastline (Caburlotto *et al.*, 2008). A second strain, VP_eVE_{pan}2, isolated on the same date and in the same site, but from a plankton sample, also carried the *tdh* and *orf8* genes and was positive at group-specific PCR.

Partial sequencing of the VP_eVE_{pan} *orf8* amplification product revealed identity (100% similarity) with the reported sequences of the gene *orf8* of pandemic strains. Moreover, bidirectional sequencing (GeneBank, accession number bankit1122717 FJ194945) of the two VP_eVE_{pan} environmental strain amplicons obtained with the *tdh451* primers (Okura *et al.*, 2003) showed that the *tdh* genes presented high similarity (96–98%) with respect to the previously reported sequences (Yoh *et al.*, 1991) of the four *tdh* gene copies encoding TDH (*tdh1*, *tdh2*, *tdh3* and *tdh4*) (Figure 2). The differences in the genomic sequence resulted in a difference of at least three amino acids in the corresponding TDH protein structure from VP_eVE_{pan}

and VP_eVE_{pan}2, but the Wagatsuma test demonstrated that they are functional and show the characteristic Kanagawa phenomenon in blood agar plates (Sakazaki *et al.*, 1968; Nishibuchi and Kaper, 1990).

Lipopolysaccharide (O) and capsular (K) serotypes of VP_eVE_{pan} and VP_eVE_{pan}2 were determined using specific commercial antisera and were O5:K68 for the first strain and O11:K40 for the second strain. Both serotypes are included in the list of the 'pathogenic O and K antigen combinations' (Honda *et al.*, 2008), but are not included in the so-called 'pandemic group' (Nair *et al.*, 2007).

Molecular typing of the two environmental strains carrying the pandemic markers was conducted using two different typing methods, namely pulsed-field gel electrophoresis and ERIC-PCR. As shown in Figure 3, both the environmental strains, VP_eVE_{pan} and VP_eVE_{pan}2, showed identical pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus-PCR profiles. Moreover, the profiles proved to be very similar to those of the strains AN 2416 and QM 97097 used as pandemic reference strains (serotype O3:K6), with 85% similarity, which indicates that the four analyzed strains belong to the same cluster (Figure 3).

In this paper, we described the serological and molecular characteristics of the previously reported strain, VP_eVE_{pan}, and of another strain, VP_eVE_{pan}2, contemporary isolated in a plankton sample from the same geographic site (Caburlotto *et al.*, 2008). To the best of our knowledge, these strains, isolated in the Italian coastal environment directly from marine water and plankton, are the first environmental strains of *V. parahaemolyticus* carrying genetic markers associated with the 'pandemic strains' (*tdh*, *orf8*, *toxRS/new*) isolated in Europe.

Figure 1 Agarose gel electrophoresis of *V. parahaemolyticus* strain VP_eVE_{pan} PCR-amplified fragments: (a) *orf8* (lane 1, 369 bp); (b) *tdh* (lane 1, 263 bp); (c) *toxRS* (lanes 1 (500 ng DNA) and 2 (150 ng DNA), 651 bp); lanes A2, B2 and C3 are positive controls using the DNA of the reference O3:K6 pandemic strain AN2416, *tdh*(+), *trh*(–), *ttss2*(+), *methyltransferase* (VP0394)(+), *GS-PCR*(+) kindly provided by Dr RYC Kong of the University of Hong Kong.

Figure 2 Partial sequence of VPeVEpan *tdh* gene (a) and protein (b) as compared with the nucleotide sequences of genes *tdh1*, *tdh2*, *tdh3* and *tdh4* and their corresponding proteins.

Figure 3 Pulsed-field gel electrophoresis (a) and enterobacterial repetitive intergenic consensus-PCR (b) profiles of the two environmental *V. parahaemolyticus* strains, VPeVEpan and VPeVEpan2, carrying pandemic markers compared with AN2416 and QM97097, the two reference pandemic O3:K6 strains.

Although the environmental strains described here do not belong to the serotypes more frequently included in the ‘pandemic group’ (Nair *et al.*, 2007), their molecular profiles obtained both by pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus-PCR are identical to one another and very similar (85% similarity, strains belonging to the same cluster) to those of two Asian pandemic strains showing the O3:K6 serotype. Moreover, the genotyping of the two environmental strains permitted the detection of three virulence-related genes, an associated DNA methyltransferase on a 22.79-kb pathogenicity island-like element on *V. parahaemolyticus* chromosome 1 (Wang *et al.*, 2006) and *T3SS2* genes (*vscC2*, *vopP*) involved in the cytotoxicity and enterotoxigenicity of *V. parahaemolyticus* (Park *et al.*, 2004). These genes are associated with clinical strains, but are hardly ever (*MTase* gene) or never (*T3SS2* genes) detected in environmental strains.

The presence, in strains isolated from the marine environment, of genetic pandemic markers and virulence genes normally associated with clinical strains indicates that these *V. parahaemolyticus* strains might constitute a reservoir of virulence genes that could spread in the marine ecosystem.

For this reason, they should be seen as a public health concern and as a possible risk for human health.

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