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Molecular characterization and drug susceptibility of non-O1/O139 *V. cholerae* strains of seafood, environmental and clinical origin, Italy

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ABSTRACT

Toxigenic and antimicrobial susceptibility patterns and genetic relatedness of 42 non-O1/O139 *V. cholerae* strains, the majority of them isolated from seafood and marine water of the Adriatic sea, Italy, and 9 clinical strains, two of which with seawater of the Adriatic as the source of infection, were studied. All strains had *hly*A El Tor gene but lacked *ctx*A gene. Four and two isolates, respectively, also had *stn/sto* and *tcp*A Class genes. More than 90% of strains showed susceptibility to cefotaxime, ciprofloxacin, chloramphenicol, tetracycline, trimethoprim + sulfamethoxazole and intermediate or full resistance to tetracycline and erythromycin. Six strains of seafood and clinical source were multi-drug resistant. PFGE analysis allowed to type all the strains with 50 banding patterns. Twenty-one strains, 11 and 8 from seafood and seawater, respectively, and 2 of clinical origin, were grouped into 9 different clusters. We report the presence of toxigenic and multidrug resistant non-O1/O139 *V. cholerae* strains in Adriatic, some of which genetically related, and support that they represent a potential reservoir of toxin and antibiotic resistance genes.

1. Introduction

Vibrio cholerae bacteria are ubiquitous in the aquatic environment and infections due to these microorganisms are strongly associated with water or raw food ingestion or water exposure (Chen et al., 2015; Janda et al., 1988). Although *V. cholerae* O1 is the notorious microorganism that can cause epidemic or pandemic diarrheal disease, emerging intestinal and extra-intestinal infections due to non-O1/O139 *V. cholerae* have become another not negligible problem (CDC, 2016). *V. cholerae* non-O1/O139 strains can carry the gene for cholera toxin (*ctx)* and other toxin genes, including those for toxin-coregulated pilus (*tcp*A), heat stable enterotoxin (*stn/sto)*, and hemolysin (*hly*A) (Chen et al., 2015; Janda et al., 1988; Ottaviani et al., 2009; Rivera et al., 2001; Sharma et al., 1998). However, many cases of intestinal and extra-intestinal infection are linked to non-toxigenic strains, so the exact mechanism of non-O1/O139 *V. cholerae* pathogenesis has yet to be clarified (Chen et al., 2015; Farina et al., 2010; Ottaviani et al., 2011, 2009). In the United States around 40 cases of *V. cholerae* non-O1/O139 infections are annually reported (CDC, 2016). In Europe, the lack of mandatory notification systems for *Vibrio*-associated illnesses diverse from those caused by *V. cholerae* O1/O139 prevents accurate estimates of the number of infections. However, outbreaks caused by *Vibrio cholerae* non-O1/O139 are increasing overtime and this is probably linked to the progressive rise in sea surface temperature (Le Roux et al., 2015). Although there are no official recommendations on antibiotic therapy for *V. cholerae* non-O1/O139 infections, an early administration of antibiotics can prevent a fatal outcome of extra-intestinal diseases (Engel et al., 2016; Feghali and Adib, 2011; Lu et al., 2014; Ottaviani et al., 2011; Petsaris et al., 2010). In the last decade, the presence of non-O1/O139 *V. cholerae* in the Adriatic sea and non-O1/O139 *V. cholerae* infections, for which epidemiological informations had identified seawater of the Adriatic sea as the likely source of infection, have been reported

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(Ottaviani et al., 2011, 2009). In this work we studied toxigenic, antibiotic-resistance and genetic patterns of *V. cholerae* non-O1/O139 strains isolated in the last decade from various sources, most of them linked to the Adriatic sea. The results were analyzed to define the relationships among these strains and gain preliminary information on the potential of the Adriatic sea as environmental reservoir of toxin and antibiotic resistance genes.

2. Material and methods

2.1. Strain collection

We analysed 51 *V. cholerae* non-O1/O139 strains collected from 2003 to 2014 in Italy. Of these, 37 strains were isolated from seawater or seafood, five strains from fresh water and nine from clinical samples (Table 1). Regard the origin of seafood and seawater strains, 31 were from Adriatic sea, 2 from Mediterranean sea and 4 from Asia. Seven clinical strains were from faeces of Italian diarrhoeal patients. In these cases, epidemiological information, collected through patient interviews, indicated seafood as the likely source of infection, but no more details were available about seafood origin. Two clinical strains were from subcutaneous tissue of patients affected by necrotizing fasciitis, after direct exposure to seawater. In these cases, epidemiological information indicated the Adriatic sea as the likely source of infection (Table 1).

2.2. Strains identification

Strains were biochemically identified by a standardized protocol (Noguerola and Blanch, 2008; Ottaviani et al., 2003) and also confirmed by PCR analysis of 16S-23S rRNA intergenic spacer regions of *V. cholerae* (Chun et al., 1999).

2.3. Molecular analysis

Amplification by PCR of the *ctx*A, Classical (Class) and El Tor (ET) *tcp*A and *hly*A, *stn*/*sto* genes was performed using primers and PCR conditions previously described (Ottaviani et al., 2009; Rivera et al., 2001). Analysis of chromosomal DNA restriction patterns by PFGE was performed according to the PulseNet standardized protocol for *V. cholerae* (Cooper et al., 2006). *Sfi*I (5-GGCCNNN-3) was used as primary enzyme to perform PFGE analysis. Both enzymes *Sfi*I and *Not*I (5-GCGGCCGC-3) were used where the PFGE patterns obtained with the primary enzyme for two or more isolates were indistinguishable.

2.4. Antimicrobial susceptibility

Antimicrobial susceptibility to 14 antimicrobial agents was determined by the disk diffusion method, according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010): (AMP 10 μg); amoxicillin + clavulanic acid (AMC 20/10 μg), cefotaxime (CTX 30 μg), ciprofloxacin (CIP 5 μg), cloramphenicol (C 30 μg), colistin sulphate (CT 10 μg), erythromycin (E 15 μg), gentamicin (CN 10 μg), meropenem (MER 10 μg), tetracycline (TE 30 μg), trimethoprim + sulfamethoxazole (ST 23.75/1.25 μg), nalidixic acid (NA 30 μg), streptomycin (S 10 μg), kanamicin (K 30 μg). The CLSI interpretative criteria for disk diffusion susceptibility testing of *Vibrio* spp. (CLSI, 2010) when available or, in alternative, those according to the manufacturer's instructions, were used.

2.5. Reference strains

V. cholerae O1 classical biotype ATCC 9459 was used as the positive control for PCR amplification of *tcp*AClass, *hyl*AClass and *ctx*A genes. The previously characterized field strains, *V. cholerae stn/sto* positive (VCST) and *V. cholerae* O1 El Tor biotype (VCET) from the authors' collection (Ottaviani et al., 2009), were used, respectively, as positive controls for PCR amplification of the *stn/sto*, and *hly*AET and *tcp*AET genes (Table 1).

2.6. Statistical analysis

Prevalence data were examined by a chi-square test ($\chi 2$) and a probability value (P) ≤ 0.05 was regarded as statistically significant. For PFGE analysis, tiff images were analysed using BioNumerics version 7.6.2 (Applied Maths NV). Pattern similarity (PS) was determined by the Dice coefficient, with a 1.0–1.5% tolerance window, and a dendrogram was constructed using the unweighted-pair group method (UPGMA). Clusters were defined on the basis of the 80% similarity cut-off (Ottaviani et al., 2013). Indistinguishable patterns were visually confirmed.

3. Results and discussion

3.1. Toxin genes

Regarding the distribution of toxigenic patterns (TP)s, 45/51 (88%), 4/51 (8%), 2/51 (4%) strains had TP1 (*hly*AClass-; *hly*ET+;*ctx*A-; *tcp*AClass-*tcp*AET-;*stn/sto-)*, TP3(*hly*AClass-;*hly*ET+;*ctx*A-;*tcp*A-Class-; *tcp*AET-;*stn/sto+)* and TP2 (*hly*AClass-; *hly*ET+;*ctx*A-*tcp*AClass+; *tcp*AET-*; stn/sto)*, respectively (Table 2). All strains had *hly*AET gene but lacked *hly*AClass, *ctx*A, *tcp*AET genes. Four strains (8%), of which 1 and 3 isolated, respectively, from Asian seafood in 2005 and seawater of the North Adriatic in 2011, had the *stn/sto* gene (Table 2). Two strains (4%), one isolated in 2009 from subcutaneous tissue of a patient affected by necrotizing fasciitis and the other from freshwater in 2010, possessed the *tcp*A Class gene (Table 2).

3.2. Antimicrobial susceptibility

More than 90% of the isolates were susceptible to CIP, C, TS, TE, CTX (Table 3). Towards CT, AMP, NA, AMC, K, S, CN, E, MER resistance was found in 96% (n = 49/51), 22% (n = 11/51), 12% (n = 6/51), 8% (n = 4/51), 6% (3/51), 4% (2/51), 4% (n = 2/51), 2% (n = 1/51), 2% (n = 1/51) of the isolates, respectively, and intermediate resistance in 2% (n = 1/51), 41% (n = 21/51), 0% (n = 0/51), 35% (n = 18/51), 20% (n = 10/51), 53% (n = 27/51), 31% (n = 16/51), 92%(n = 47/51), 26% (n = 13/51), respectively (Table 3). No significant difference was found in the distribution of resistance, with respect to origin, source or year of strain isolation (Supplementary Table S3). Six strains, 4 from seafood (TO3, TO1, 35643, 2570/2.2) and 2 from clinical samples (38 098, 44 868), showed resistance to more than two different classes of antibiotics and therefore were considered to be multi-drug resistant (Table 3). The seafood strain 35643 that showed AMP/CT/MER multidrug resistant pattern also had *stn/sto* gene (Supplementary Table S3). Moreover, the clinical strain 55 was susceptible only to AMP and showed intermediate resistance toward all other antibiotics (Supplementary Table S3).

3.3. PFGE analysis

The PFGE characterization allowed to type all the 51 strains analyzed in this study, which exhibited 50 different PFGE patterns (two strains shared the same pattern) (Table 2, Fig. 1). Twenty-one strains, 11 and 8 from seafood and seawater, respectively, and 2 of clinical origin, were grouped into 9 different clusters (C)s (Fig. 1). C I included strains 38 631 and 30 116, both isolated in Central Italy, the first in 2008 from fresh water, the second in 2009 from seafood