Detection and characterisation of potentially pathogenic species of Vibrio *in the Vibrata river, Abruzzo Region, Italy*

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Keywords
PCR,
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River,
Vibrio spp.,
Virulence genes.

Summary
This study aimed to isolate, define the genetic profile, assess potential pathogenicity and evaluate the seasonal distribution of *Vibrio cholerae, Vibrio parahaemolyticus*, and *Vibrio vulnificus* strains isolated from the Vibrata river (Abruzzo Region, Italy) during a monitoring period of one year. Detection was performed according to ISO/TS 21872-1-2:2007. Species identification and characterisation were achieved using molecular methods. *Vibrio* spp. were detected in 50% (23) of the water samples. In particular, *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* were isolated in 18 (39.1%), 4 (8.7%), and 2 (4.3%) samples, respectively. All *V. parahaemolyticus* strains were *tdh* gene negative, 75% were positive for *trh* gene. In 30 *V. cholerae* isolates, the polymerase chain reaction (PCR) assay for detecting virulence and regulatory genes (*ctxA, toxR, tcpA, ompU, hlyA, tcpI, zot*, and *stn/sto*) revealed 6 genotypes associated to different levels of pathogenicity. Pulsed-field gel electrophoresis (PFGE) characterisation of the *V. cholerae* strains identified 13 different pulsotypes. A greater degree of similarity was shown for strains isolated in the same period of the year. Results of our study reveal a potential health risk associated with the waters of the Vibrata river, which are used for irrigation and next to the swimming areas of Abruzzo coastline.

Identificazione e caratterizzazione di Vibrio *potenzialmente patogeni nelle acque del torrente Vibrata (Abruzzo, Italia)*

Parole chiave
Geni di virulenza,
PCR,
PFGE,
Torrente,
Vibrio spp.

Riassunto
Il presente studio ha avuto l'obiettivo di identificare, caratterizzare e valutare l'andamento stagionale di ceppi di *Vibrio cholerae, Vibrio parahaemolyticus* e *Vibrio vulnificus*, isolati da campioni d'acqua prelevati nel torrente Vibrata (Abruzzo, Italy) durante un periodo di monitoraggio di un anno, al fine di definirne il profilo genetico e la potenziale patogenicità. La ricerca di *Vibrio* spp. è stata eseguita con isolamento su piastra (ISO/TS 21872-1-2:2007), l'identificazione di specie, la ricerca dei geni di patogenicità e la caratterizzazione delle specie *V. cholerae, V. parahaemolyticus* e *V. vulnificus* mediante tecniche molecolari. Dei 46 campioni analizzati, il 50.0% (23) è risultato contaminato da *Vibrio* spp. In particolare, sono stati isolati *V. cholerae, V. parahaemolyticus* e *V. vulnificus* rispettivamente in 18 (39.1%), 4 (8.7%) e 2 (4.3%) campioni esaminati. Tutti gli isolati di *V. parahaemolyticus* sono risultati negativi per la presenza del gene *tdh* mentre il 75% di essi è risultato positivo per il gene *trh*. La ricerca, mediante PCR multiplex, dei geni di patogenicità *ctxA, toxR, tcpA, ompU, hlyA, tcpI, zot* e *stn/sto* sui 30 isolati di *V. cholerae* ha permesso di rilevare sei diversi genotipi corrispondenti a diversi potenziali di patogenicità. I profili dei ceppi di *V. cholerae* ottenuti mediante PFGE hanno evidenziato la presenza di 13 pulsotipi diversi anche se un maggior grado di similarità è stato evidenziato tra i ceppi isolati nello stesso periodo dell'anno. Questi risultati rivelano un potenziale rischio sanitario associato alle acque del torrente Vibrata, spesso utilizzate per irrigazione di colture e prossime alle zone balneabili della costa abruzzese.

Introduction

The *Vibrio* genus includes more than 100 species (Romalde *et al.* 2014); in light of the wide genetic variability of the group this is a number which probably will increase. *Vibrio* species are one of the most representative toxin producer microorganisms of aquatic environments, especially in coastal, brackish, and estuarine waters. The Italian Food and Drug Administration (FDA-BAM 2004) considers 9 species of *Vibrio* as potentially pathogenic to humans. Amongst them, *Vibrio cholerae* (serogroups O1 and O139), *Vibrio parahaemolyticus*, and *Vibrio vulnificus* are considered the most commonly associated with seafood-borne infections.

Strains of *V. cholerae* belonging to the serogroups O1 and O139 are the causative agent of cholera, an epidemic diarrheal disease, which still affects millions of people in most developing countries of Asia, Africa, and Latin America. Besides the toxigenic O1 and O139 serogroups, *V. cholerae* strains belonging to more than 200 serogroups are widespread in aquatic environments. A number of reports have demonstrated that some strains of these serogroups can cause diarrhea or local infections including otitis and wound infections (Octavia *et al.* 2013, Shirmeister *et al.* 2014).

Vibrio parahaemolyticus is recognised throughout the world as the leading causal agent of human gastroenteritis resulting from the consumption of raw seafood. Enteropathogenic strains of *V. parahaemolyticus* generally produce a thermostable direct hemolysin (TDH) and/or a TDH-related hemolysin (TRH). The genes *tdh* and *trh* code for TDH and TRH, respectively (Cantet *et al.* 2013).

Vibrio vulnificus is able to cause severe or life-threatening infection in susceptible individuals. The spectrum of illness can vary from gastroenteritis to 'primary sepsis' and necrotising fasciitis. The case-fatality rate has been reported to exceed 50% in primary sepsis. Infection may result from consuming or handling contaminated seafood (usually shellfish such as oysters) or from exposing open wounds or broken skin to contaminated salt or brackish water. The presence of pathogenic *Vibrio* spp. in estuarine and coastal waters of the Mediterranean Sea has been previously reported (Gugliandolo *et al.* 2009).

The hydrographic basin (116 km^2) of the Vibrata river is located in the northern part of the Abruzzo Region (central Italy), and extends eastward and upright to the Adriatic shoreline (Figure 1). Ten waste-water treatment plants are located along the basin. The river is designated as a Nitrate Vulnerable Zone according to European legislation, and was classified as having a 'bad' ecological status in 2012 (Di Lorenzo *et al.* 2012, Regione Abruzzo-Monitoraggio acque superficiali, 2010-2012). The problems of the Vibrata are due to an inadequate water flow because of its use for irrigation and industries, and because of the strong anthropisation of the area and poor self-purifying capacity of the river. Urban sewage is often the main source of microbial contamination of aquatic environments posing a risk to human health for recreational activities involving water, and limiting the use of water as an economic resource.

Between the 17th and the 25th of August, 2010, a high mortality rate was recorded in fishes of the Vibrata river. During the same time period, many cases of human gastroenteritis were also reported in the area (unpublished data).

A monitoring programme was then implemented in order to check faecal, chemical, and biological

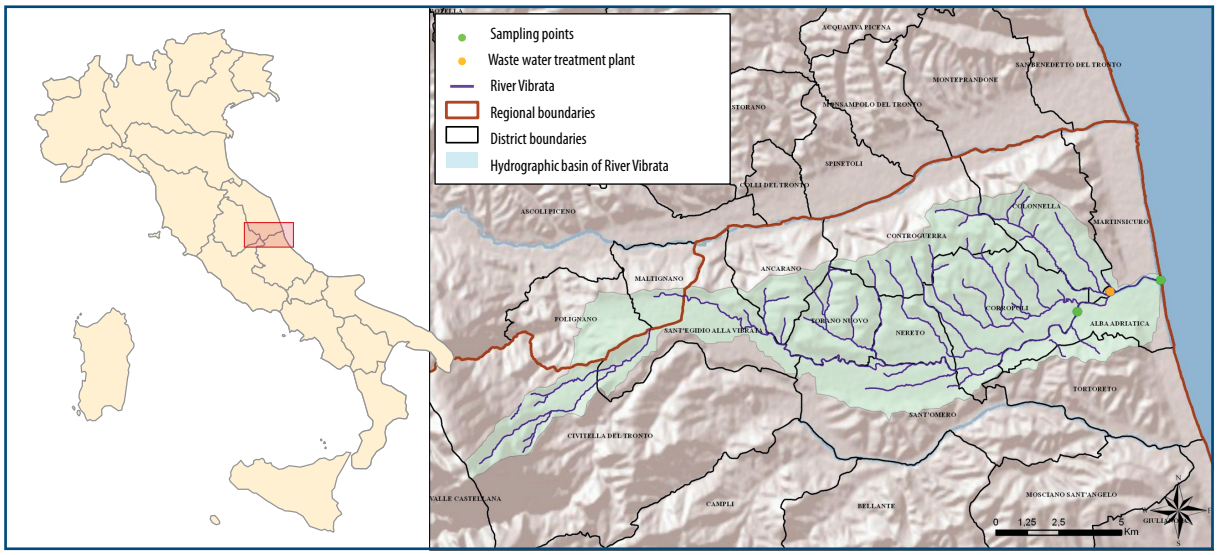


Figure 1. *The hydrographic basin of the River Vibrata located in the northern part of the Abruzzo Region (central Italy).*

contaminants in the river. Different human pathogens have been detected in water samples, including *Vibrio* spp.

This study aimed to isolate, characterise, define the genetic profile, assess potential pathogenicity and evaluate the seasonal distribution of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* strains in water samples of the Vibrata river.

Despite statistical correlations between genotypes and strains of clinical or environmental origin, pathogenetical studies in *V. vulnificus* have not yielded genotypic markers that predict virulence unequivocally (Reynaud *et al.* 2013). To date, no available molecular marker has sufficient resolving power to categorise with absolute certainty the pathogenicity of a *V. vulnificus* strain. For this reason, the focus of studies on *V. vulnificus* was on detection only.

Materials and methods

Sampling

From August 2010-August 2011 a total of 46 samples (1 litre), 23 upstream and 23 downstream (20 meters from the estuary) were collected from the waste-water treatment plant in the town of Alba Adriatica (Teramo, Italy).

Sampling was performed according to the Decree of the Italian Ministry of Health[1].

The samples were then placed in a refrigerated cool box at 4 ± 1°C, sent to the laboratory, and analysed within 24 hours.

Microbiological analysis

Analysis of the water samples was performed using the membrane filter technique. Each 1-litre sample was filtered through 0.45 µm diameter membrane (ISO 8199:2005[2]) and analysed according to ISO/TS 21872-1:2007 ISO/TS 21872-2:2007[3]. Briefly, after filtration, 100 mL of alkaline peptone water (APW) was added to the membrane and incubated at 37 ± 1°C for 6 hours for its first enrichment and cell growth. An aliquot (1 mL) was inoculated into 9 mL of APW (second enrichment) and incubated at 37 °C for 18 hours. At the same time, an aliquot was streaked in Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) and modified Cellobiose Polymyxin B Colistin (mCPC) agar. These media were then incubated at 37 ± 1°C for 24 ± 3 hours. A second passage on TCBS and mCPC agar was done starting from the second enrichment broth. A minimum of five typical colonies were picked from each selective media and cultured on Saline Nutrient Agar (SNA) in order to perform species identification through multiplex polymerase chain reaction (PCR).

Presumptive *V. cholerae* colonies were sent to the Istituto Superiore di Sanità (ISS) to confirm species identification and to perform serological test with O1/O139 antisera.

Molecular analysis

Species identification

Species identification was performed using multiplex PCR targeting a housekeeping gene that encodes heat shockprotein 40 (*dnaJ*) according to Nhung and colleagues (Nhung *et al.* 2007). This method has been used both to identify the colonies that are biochemically recognised as *Vibrio* spp., and to screen the first enrichment broth (APW) in which the membrane filter was placed.

The method used to extract DNA diverged from the reference protocol. Briefly, each isolate, that had been biochemically identified as *Vibrio* spp. and cultured on SNA for 24 hours at 37 ± 1°C, was suspended in 500 µL of Tris-EDTA buffer (50 mM Tris-HCl pH 8.0 - 1 mM EDTA pH 8.0), and then centrifuged (Eppendorf 5402 rotor F-45-18-11, Milan, Italy) at 13400 rpm for 5 minutes. The pellet was then reconstituted in 300 µL of Tris-EDTA, heated at 100 ± 1 °C for 15 minutes and transferred to at 4 ± 1°C. After centrifugation at 13400 rpm for 5 minutes, the supernatant containing the DNA was transferred to a clean test tube and stored at 4 ± 1°C.

When the method was used to screen the enrichment broth, few more steps were added before suspending the pellet in 500 µL of Tris-EDTA buffer. Briefly, 1 mL of the second enrichment broth was centrifuged at 13400 rpm for 6 minutes, the pellet was then washed in 1 mL of Tris-EDTA buffer, centrifuged at 13400 rpm for 6 minutes and then suspended in 500 µL of Tris-EDTA buffer.

The primers (MWG, Ebersberg, Germany) used for *dnaJ* amplification are listed in Table I. *Vibrio alginolyticus* (ATCC 17749), *V. cholerae* (ATCC 9458), *V. mimicus* (ATCC 33653), *V. parahaemolyticus* (ATCC 17802), and *V. vulnificus* (ATCC 27562) were added to each PCR run as positive control; nuclease free

[1] Decreto 30/03/2010. Definizione dei criteri per determinare il divieto di balneazione, nonché modalità e specifiche tecniche per l'attuazione del decreto legislativo 30 maggio 2008, n. 116, di recepimento della direttiva 2006/7/CE, relativa alla gestione della qualità delle acque di balneazione. *Off J Suppl 97*, **119**, 24/05/2010.

[2] ISO 8199. 2005. Water quality - General guidance on the enumeration of micro-organisms by culture. International Organization for Standardization. Geneva.

[3] ISO/TS 21872-1. 2007. Microbiology of food and animal feeding stuffs - Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. - Part 1: Detection of *Vibrio parahaemolyticus* and *Vibrio cholerae*. ISO/TS 21872-2. 2007 Part 2: Detection of species other than *Vibrio parahaemolyticus* and *Vibrio cholerae*. International Organization for Standardization. Geneva.

Table I. *Primers used in this study.*

Primer	Primer sequence	Gene target	Amplicon length
VM-F Univ	5'- CAG GTT TGY TGC ACG GCG AAG A- 3'	*dnaJ*	
VC- R	5'- AGC AGC TTA TGA CCA ATA CGC C- 3'	*dnaJ* VC[a]	375 bp
VP-R	5'- TGC GAA GAA AGG CTC ATC AGA G- 3'	*dnaJ* VP[b]	96 bp
VV-R	5'- GTA CGA AAT TCT GAC CGA TCA A- 3'	*dnaJ* V V[c]	412 bp
VM-R	5'- YCT TGA AGA AGC GGT TCG TGC A- 3'	*dnaJ* VM[d]	177 bp
V.al2-MmR	5'- GAT CGA AGT RCC RAC ACT MGG A- 3'	*dnaJ* VV[e]	144 bp
Trh F	5'TTG GCT TCG ATA TTT TCA GTA TCT 3	*Trh* VP[b]	500 bp
Trh R	5' CAT AAC AAA CAT ATG CCC ATT TCC G 3'		
Tdh F	5' GTA AAG GTC TCT GAC TTT TGG AC 3'	*Tdh* VP[b]	269 bp
Tdh R	5'TGG AAT AGA ACC TTC ATC TTC ACC 3'		
CtxA F	5' CGG GCA GAT TCT AGA CCT CCT G 3'	*Ctx*A VC[a]	564 bp
CtxA R	5' CGA TGA TCT TGG AGC ATT CCC AC 3'		
OmpU F	5'ACG CTG ACG GAA TCA ACC AAA G 3'	*Omp*U VC[a]	869 bp
OmpU R	5' GCG GAA GTT TGG CTT GAA GTA G 3'		
Zot F	5' TCG CTT AAC GAT GGC GCG TTT T 3'	*Zot* VC[a]	947 bp
Zot R	5' AAC CCC GTT TCA CTT CTA CCC A 3'		
VC-ToxR F	5' CCT TCG ATC CCC TAA GCA ATA C 3'	*Tox*R VC[a]	779 bp
VC-ToxR R	5' AGG GTT AGC AAC GAT GCG TAA G 3'		
TcpA F	5' CAC GAT AAG AAA ACC GGT CAA GAG 3'	*Tcp*A VC[a]	451 bp (El Tor)
TcpA R1	5' CGA AAG CAC CTT CTT TCA CGT TG 3'		620 bp (Clas)
TcpA R2	5'TTA CCA AAT GCA ACG CCG AAT G 3'		
TcpI F	5'TAG CCT TAG TTC TCA GCA GGC A 3'	*Tcp*I VC[a]	862 bp
TcpI R	5' GGC AAT AGT GTC GAG CTC GTT A 3'		
HlyA F1	5' GGC AAA CAG CGA AAC AAA TAC C 3'	*Hly*A VC[a]	481 bp (El Tor)
HlyA F2	5' GAG CCG GCA TTC ATC TGA AT 3'		738/727 bp (Clas)
HlyA R	5' CTC AGC GGG CTA ATA CGG TTT A 3'		
Stn/sto F	5'TCG CAT TTA GCC AAA CAG TAG AAA 3'	*Stn/sto* VC[a]	172 bp
Stn/sto R	5' GCT GGA TTG CAA CAT ATT TCG C 3'		

F = forward primer; R = reverse primer; bp = base pair. [a] *Vibrio cholerae*; [b] *Vibrio parahaemolyticus*; [c] *Vibrio vulnificus*; [d] *Vibrio mimicus*; [e] *Vibrio alginolyticus*.

water (Ambion®- Life Technologies, Monza, Italy) was added as negative control.

A reaction mixture containing 12.5 µL of PCR master mix (Promega, Madison, USA), 1 µM of each primer (10 µM), nuclease free water and 2 µL of DNA was prepared for a final volume of 25 µL. The reaction was performed with the thermal cycler Applied Biosystem 9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: one cycle of 3 minutes at 96 °C, 35 cycles of 0.5 minute at 95 °C, 0.5 minute at 60 °C, and 1 minute at 72 °C, a final cycle of 7 minutes at 72 °C, and storage at 4 ± 1°C.

PCR products were analysed by electrophoresis in a 2% agarose gel (Agarose D-1- LOW EEO, Eppendorf, Milan, Italy) and stained with SYBR® Safe DNA gel stain (2.5 µL/100 mL) (Invitrogen, Eugene, OR, USA). Bands were visualised using a UV trans-illuminator and photographed using the programme Chemilmager 5500. AmpliSize™ Molecular Ruler 50-2000 bp Ladder (Sigma-Aldrich, St Louis, USA) was used as a molecular weight marker.

Virulence gene detection

Vibrio parahaemolyticus

Vibrio parahaemolyticus strains were tested for the virulence genes (*tdh* and *trh*) according to the protocol set out by Bej and colleagues (Bej *et al.* 1999), with one minor change: in this study single instead of multiplex PCRs were used.

The DNA extraction process was performed as previously described. Primers (MWG, Ebersberg, Germany) used for *tdh* and *trh* amplification are listed in Table I.

ATCC 17802 and ATCC 43996 strains of *Vibrio*

parahaemolyticus were added to each PCR run as positive controls for *trh* and *tdh*, respectively. Nuclease-free water was added as negative control.

A reaction mixture containing 25 µL of PCR master mix (Promega, Madison, USA), 1 µL of each primer (50 µM), 3 µL of $MgCl_2$ (25 mM), nuclease-free water, and 2 µL of DNA was prepared. The final volume was 50 µL. Reactions were performed with a thermal cycler Applied Biosystem 9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: one cycle of 10 minutes at 94 °C, 30 cycles of 1 minute at 94 °C, 1 minute at 58 °C, and 2 minutes at 72 °C, a final cycle of 7 minutes at 72 °C and then storage at 4 ± 1°C.

PCR products were analysed by electrophoresis in a 1.8% agarose gel (Agarose D-1- LOW EEO, Eppendorf, Milan, Italy). Bands were visualised as previously described.

Vibrio cholerae

Vibrio cholerae strains were tested for genes encoding a protein for regulation of virulence (VC-*tox*R), an hemolysin (*hly*A), the cholera toxin (*ctx*A), the toxin-coregulated pilus (*tcp*A), a homolog of methyl-accepting chemotaxis protein (*tcp*I), an outer membrane protein (*omp*U), a zonula occludens toxin (*zot*), and a heat-stable enterotoxin (*stn/sto*) according to the protocol set out by Rivera and colleagues (Rivera *et al.* 2001), with one minor change: in this study single instead of multiplex PCRs were used. The DNA extraction process was performed as previously described. Primers (MWG, Ebersberg, Germany) are listed in Table I.

Vibrio cholerae (Ogawa El Tor 77/65) was added to each PCR run as a positive control. Nuclease-free water was added as a negative control. For *stn* gene, it was not possible to have a positive control, but all the strains identified as *V. cholerae*, were sent to the Istituto Superiore di Sanità (ISS) to confirm their identification and to evaluate their pathogenicity.

The reaction mixture contained 12.5 µL of PCR master mix (Promega, Madison, USA), 1 µL of each primer (20 µM), nuclease-free water, and 3 µL of DNA. The final volume was 25 µL. The reactions were performed with a thermal cycler Applied Biosystem 9700 (Applied Maths, Saint-Martens-Latem, Belgio) under the following conditions: one cycle of 10 minutes at 94 °C, 30 cycles of 1 minute at 94 °C, 1 minute at 60 °C, and 2 minutes at 72 °C, a final cycle of 7 minutes at 72 °C and then storage at 4 ± 1 °C. The *Stn/sto* detection process was performed at an annealing temperature of 55 °C instead of 60 °C. PCR products were analysed by electrophoresis in a 1.5% agarose gel (Agarose D-1- LOW EEO, Eppendorf, Milan, Italy). Bands were visualised as previously described.

Pulsed-field gel electrophoresis (PFGE) of *V. cholerae* strains

Vibrio cholerae strains were characterised using PFGE according to the PulseNet protocol (Cooper *et al.* 2006) which includes the restriction enzymes (*Sfi*I and *Not*I) and *Salmonella* serotyped Braenderup (H9812) as the standard. Bacterial suspensions were included in agarose, lysed, washed, and digested with the restriction enzymes. The digested samples underwent electrophoresis in SeaKem® Gold agarose 1% (Lonza, Milan, Italy) in the Chef Mapper XA (BioRad Inc, CA, USA) at 6 V/cm with the following 2 block programmes: Block 1: 2 seconds - 10 seconds, 13 hours, Block 2: 20 seconds - 25 seconds, 6 hours.

The PFGE profiles were analysed using BioNumerics software version 6.6 (Applied Maths, Kortrijk, Belgium). The similarities between the macrorestriction profiles (MRPs) were calculated using the Dice coefficient (Grothues e Tümmler, 1991), applying an optimisation coefficient and band tolerance of 1.5% for both enzymes. Clustering was performed and dendrograms generated by the unweighted pair group method using arithmetic averages (UPGMA). MRPs were assigned according to Tenover and colleagues (Tenover *et al.* 1995) and Barrett and colleagues (Barrett *et al.* 2006).

Results

Microbiological analysis

Potentially pathogenic *Vibrios* were detected in 23 samples (50%) (n = 23). In particular, *Vibrio* spp. strains were identified in 14 samples collected upstream and in 9 collected downstream of the waste-water treatment plants.

Phenotypic identification led to the isolation of more than one colony for each sample, resulting in a total of 61 strains potentially belonging to *Vibrio* genus. These strains were picked from each selective media and cultured on SNA for species identification by multiplex PCR.

Molecular analysis

Species identification

When multiplex PCR for *dnaJ* gene PCR was applied on broths, 24 samples of the 46 tested were positive to *Vibrio* spp. As from 1 sample it was not possible to isolate any strain, so this sample was considered positive to molecular screening but negative to isolation.

Of the 61 biochemically identified strains as

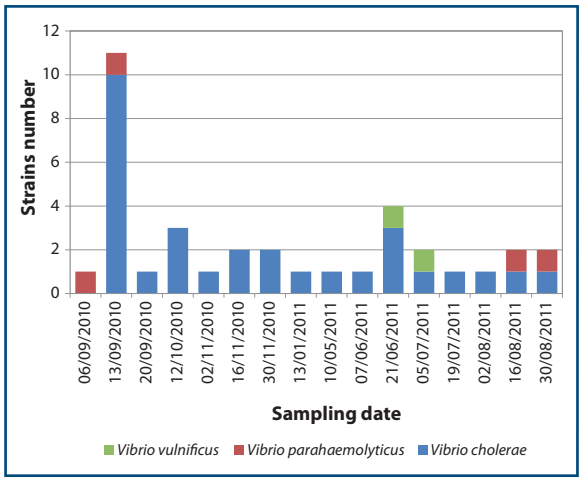


Figure 2. *Temporal distribution of potentially pathogenic* Vibrio *spp. isolates.*

Vibrio spp., multiplex PCR recognised 30 *V. cholerae* strains, 4 *V. parahaemolyticus* strains, and 2 *V. vulnificus* strains in 18 (39.1%), 4 (8.7%), and 2 (4.3%) samples, respectively. The other isolates (25) gave negative results for the expected amplicons and were collected as *Vibrio* spp. Isolate number according to sampling date are shown in Figure 2.

Serological tests of *V. cholerae* strains, performed at ISS, identified all isolates as non-O1/non- O139.

Detection of pathogenic genes

Vibrio parahaemolyticus

V. parahaemolyticus was detected in 4 of the 46 samples. Three *V. parahaemolyticus* strains (75%) were identified as *trh*-positive. Vice-versa, we did not detect isolates encoding the thermostable direct hemolysin (*tdh*) gene.

Vibrio cholerae

All the strains identified as *V. cholerae*, with biochemical methods and with *dnaJ* PCR (n = 30), were characterised according to the protocol set out by Rivera and colleagues (Rivera *et al.* 2001). Each strain was tested for the following genes: Vc-*tox*R, *hly*A, *ctx*A, *tcp*I, *tcp*A, *omp*U, *zot*, and *stn/sto*. *Vibrio cholerae* El Tor 77/65 strain was used as positive control.

PCR results showed that all the strains were *tox*R-VC positive, confirming the previous identifications of *V. cholerae*. Moreover, all the tested strains gave a *hly*A amplicon of 481 bp, meaning that they belonged to biotype El Tor. Even if all the isolates resulted negative for the *stn* toxin, a wide range of variability emerged in the pathogenic profiling. Among the 30 isolates, 6 different pathogenic patterns were found. The 6 pathogenic patterns are showed in Table II.

Pulsed-field gel electrophoresis

Of the 30 strains tested, only 22 were typeable with the restriction enzymes (*Sfi*I and *Not*I). Both enzymes displayed the same discriminatory power, allowing us to obtain 13 macrorestriction profiles for each one. Genetic relationship of the 13 pulsotypes, obtained from the combined analysis of MRPs, is graphically represented in the dendrogram in Figure 3. Pulsotypes can be grouped into 3 clusters (I, II, III): Cluster I, including almost potentially pathogenic strains, contained isolates sampled in Summer 2010 and 2011. Clusters II and III contain strains isolated between September-October 2010 and in November 2010, respectively.

Discussion

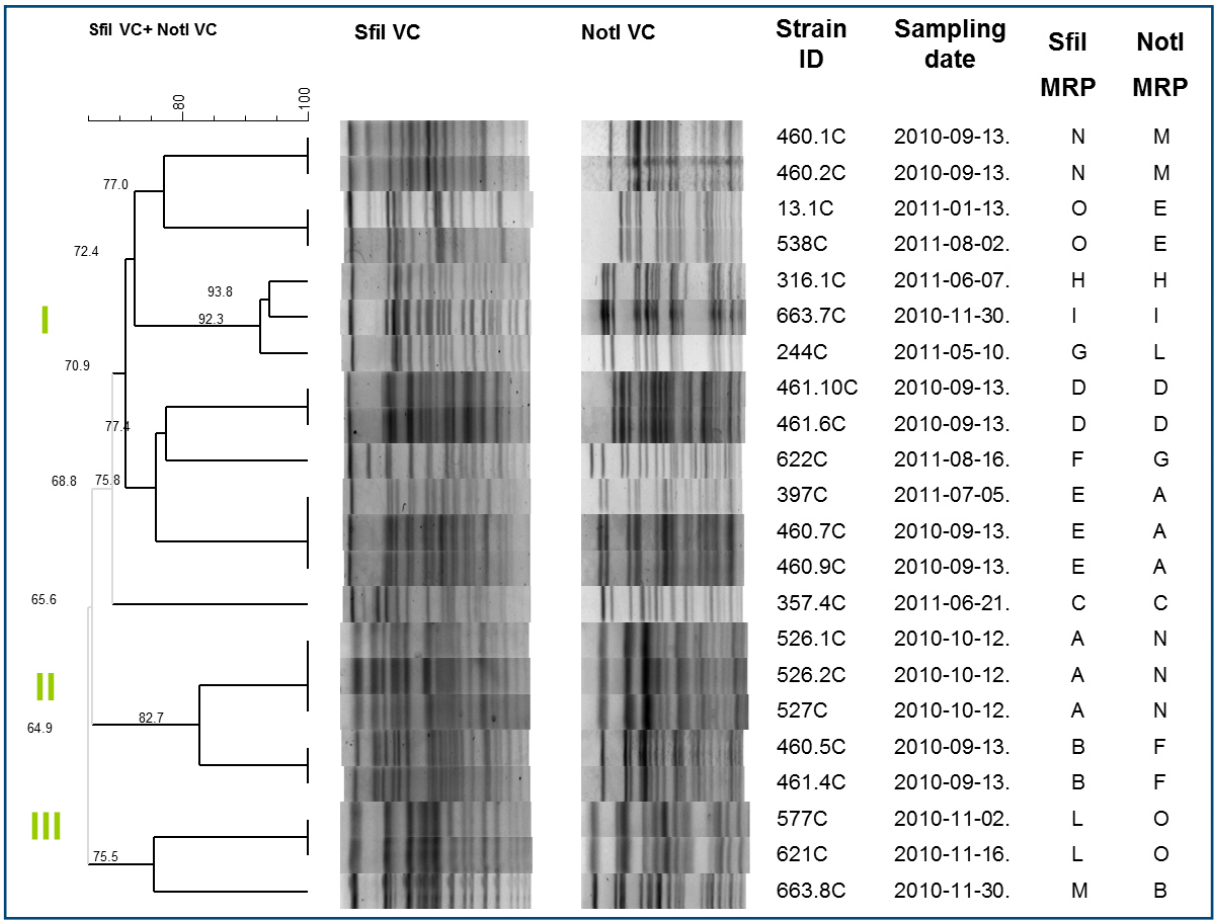
This study aimed to isolate, characterize, and evaluate the seasonal distribution of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* strains in the Vibrata river in order to define the genetic profile and assess potential pathogenicity.

Of the 46 samples, 23 (50%) were found positive to *Vibrio* spp. when tested by biochemical methods and multiplex PCR. In particular, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* strains were isolated in 18 (39.1%), 4 (8.7%), and 2 (4.3%) samples, respectively.

Table II. *Pathogenicity patterns of* V. cholerae *isolates (N = 30).*

Pathogenicity	Pathogenicity patterns								Strain number (%)
	toxR+	ctxA-	tcpA ET[a]-	tcpI-	hlyA ET[a]+	ompU-	zot-	stn/sto-	30 (n = 9)
	toxR+	ctxA-	tcpA ET[a]-	tcpI-	hlyA ET[a]+	ompU+	zot-	stn/sto-	23.3 (n = 7)
	toxR+	ctxA-	tcpA ET[a]-	tcpI+	hlyA ET[a]+	ompU-	zot-	stn/sto-	16.7 (n = 5)
	toxR+	ctxA-	tcpA ET[a]-	tcpI+	hlyA ET[a]+	ompU+	zot-	stn/sto-	3.3 (n = 1)
	toxR+	ctxA-	tcpA ET[a]+	tcpI+	hlyA ET[a]+	ompU+	zot-	stn/sto-	13.3 (n = 4)
	toxR+	ctxA-	tcpA ET[a]+	tcpI+	hlyA ET[a]+	ompU+	zot+	stn/sto-	13.3 (n = 4)

[a]ET= El Tor Biotype.



Strain ID	Sampling date	SfiI MRP	NotI MRP
460.1C	2010-09-13.	N	M
460.2C	2010-09-13.	N	M
13.1C	2011-01-13.	O	E
538C	2011-08-02.	O	E
316.1C	2011-06-07.	H	H
663.7C	2010-11-30.	I	I
244C	2011-05-10.	G	L
461.10C	2010-09-13.	D	D
461.6C	2010-09-13.	D	D
622C	2011-08-16.	F	G
397C	2011-07-05.	E	A
460.7C	2010-09-13.	E	A
460.9C	2010-09-13.	E	A
357.4C	2011-06-21.	C	C
526.1C	2010-10-12.	A	N
526.2C	2010-10-12.	A	N
527C	2010-10-12.	A	N
460.5C	2010-09-13.	B	F
461.4C	2010-09-13.	B	F
577C	2010-11-02.	L	O
621C	2010-11-16.	L	O
663.8C	2010-11-30.	M	B

Figure 3. *Cluster analysis of 22* Vibrio cholerae *strains isolated from water of Vibrata river samples based on combined* SfiI *and* NotI *macrorestriction profiles.* Similarity analysis was performed using Dice coefficients, with a 1.5% band position tolerance and 1.5% optimization. Clustering and dendrogram construction were performed using the Unweighted Pair Group Method Analysis with averages (UPGMA). MRP = Macrorestriction Profile.

The percentage of samples positive to *V. cholerae* strains found in this study was higher than that reported in similar studies on Italian rivers (Barbieri *et al.* 1999, Gugliandolo *et al.* 2009). The proportion of samples positive to *V. vulnificus* strains was also higher than that found by Gugliandolo and colleagues (Gugliandolo *et al.* 2009) and Serracca and colleagues (Serracca *et al.* 2011), in which prevalence was 0.5% and 2%, respectively. *V. vulnificus* is more frequently isolated from estuarine habitats than coastal marine waters. This is probably due to the salinity of the water, as more than 25 parts-per-thousand can have an adverse effect on the distribution of *V. vulnificus* (Serracca *et al.* 2011).

The percentage of samples positive to *V. parahaemolyticus* was instead similar to that found by Gugliandolo and colleagues in the Alcantara River in Sicily (Gugliandolo *et al.* 2009), but lower than that detected by Serracca and colleagues in the Magra river (Liguria region) (Serracca *et al.* 2011).

The one year (August 2010-August 2011) monitoring period allowed us to analyse the relationship between the isolation of potentially pathogenic *Vibrio* strains and seasonality. There was no correlation between *V. cholerae* isolation and seasonality, as the isolates were detected continuously (Figure 2). Conversely, *V. parahaemolyticus* and *V. vulnificus* strains were isolated in the warmer months (from June to September). These findings were in line with what described in the existing literature that demonstrated an increased presence of these species when the surface water exceeded 15 °C (Caburlotto *et al.* 2008, Caburlotto *et al.* 2012, Haley *et al.* 2014, Vezzuli *et al.* 2011).

On the other hand, the continuous detection of *V. cholerae* strains during the monitoring year might suggest the presence of a constant population that is able to adapt or, alternatively, has been protected from climate change. Constant isolation of *Vibrio* spp. was also reported by other authors. It was attributed to their adaptability to the adverse conditions by adhering to different substrates or planktonic organisms (Gugliandolo *et al.* 2009, Vezzulli *et al.* 2009). A recent review by Lutz and colleagues (Lutz *et al.* 2013) elucidates the complex mechanisms enabling *V. cholerae* to withstand temperature fluctuation, salinity variation, and predation.

To date, the thermostable direct hemolysin and *tdh*-related hemolysin are considered the most distinctive factors of *Vibrio parahaemolyticus* virulent strains (Ceccarelli *et al.* 2013, Chen *et al.* 2011, Shimohata *et al.* 2010).

Several studies have reported that about 90% of clinical strains contain *tdh* and *trh*, whereas, in environmental strains, these genes are typically reported in a very low percentage (0.3 to 3%) (Caburlotto *et al.* 2008, Ceccarelli *et al.*, 2013, Gutierrez *et al.* 2013, Nordstrom *et al.* 2007). Other studies on Italian rivers reveal a very low percentage for the *tdh* gene (0-0.3%) (Gugliandolo *et al.* 2009, Serracca *et al.* 2011). Our results substantiate these findings, as all of our samples did not contain *tdh* gene. In contrast, trh gene was identified in 3 of the 4 isolated strains (75%).

According to previous studies (Rivera *et al.* 2001, Shirmeister *et al.* 2014), the characterisation of *V. cholerae* strains confirmed the predominance of biotype El Tor among *V. cholerae* environmental strains, since gene encoding El Tor type hemolysin (*hly*A ET) was detected in all isolates. Furthermore, in other studies of biotypes of non-O1/non-O139 *V. cholerae*, all the strains gave an amplicon of 451 bp, confirming they belong to biotype El Tor (Bhowmick *et al.* 2009).

In this study, *Tcp*A gene, encoding toxin-coregulated pilus was detected in 26.7% (n = 8) of the isolates while *Tcp*I gene, encoding a putative methyl-accepting chemotaxis protein, was detected in 46.7% (n = 14) of the isolates. This is consistent with the 56.4% reported by Rivera and colleagues (Rivera *et al.* 2001). The *Tcp*I gene is the first gene in the TCP cluster and is associated with the synthesis of *tcp*A. It may function as a regulator to determine the virulence of the *Vibrio* pathogenicity island (Teh *et al.* 2010).

In this study, the detection of virulence genes in 30 of the non-O1/non-O139 *V. cholerae* strains produced 6 different patterns. The most frequent patterns found were: *tox*R-*hly*AET (30.0%, n = 9), *tox*R-*hly*AET-*omp*U (23.3%, n = 7), and *tox*R-*hly*AET-*tcp*I (16.7%, n = 5). Genotype *tox*R-*hly*AET-*omp*U-*tcp*I, the most frequently appearing in the Rivera's work (Rivera *et al.* 2001), was found in 1 strain only (3.3%). A greater pathogenicity was recorded in our results when compared with other studies of Italian coastal waters, in which 95% of non O1/non O139 *V. cholerae* strains showing genotype *tox*R-*hly*AET were reported (Ottaviani *et al.* 2009). In addition we detected 13.3% (n = 5) of strains containing genotype *tox*R-*hly*AET-*omp*U-*tcp*I-*tcp*A-*zot*, this pattern differs from that of pathogenic strains because of the lack of the *ctx*A gene. It is important to note that in this study the *zot* gene, encoding zonula occludens toxin, was present even when lacking the gene for the toxin *ctx*A. This finding is somewhat unexpected, since the 2 genes, together with the *ace* gene, are part of the CTX genetic element. However, similar results were obtained in other studies (Theophilo *et al.* 2006, Vital-Brazil *et al.* 2002) that analysed the prevalence of *V. cholerae* virulent strains in environmental and clinical samples isolated in Brazil. Theophilo and colleagues hypothesise that there was lost *ctx*A later integration of CTXf DNA into the *V. cholerae* chromosome. It could be the same for our strains meaning that the waters of the Vibrata river represent a risk for human health.

To date, even if serogroups O1 and O139 remain the causative agents of epidemic diarrheal disease, the current serotyping scheme of *V. cholerae* includes more than 200 serogroups that are widespread in aquatic environments. A number of reports has demonstrated that some strains of these serogroups can cause diarrheal diseases or local infections, but they do not have the ability to cause epidemic outbreaks (Bhowmick *et al.* 2009, Bidinost *et al.* 2004, Chatterjee *et al.* 2009, Faruque *et al.* 2003, Ottaviani *et al.* 2009, Shirmeister *et al.* 2014).

Non-O1/non-O139 strains isolated from different clinical cases as well as environmental cases demonstrated the ability to cause disease even in the absence of *ctx*AB and *tcp*A genes (Chatterjee *et al.* 2009, Octavia *et al.* 2013, Shirmeister *et al.* 2014).

Strains isolated in this study can be considered as potentially pathogenic and convertible to toxigenic. Colonisation of the intestinal mucosa is a fundamental step in the ability of enteric pathogens to create productive infection. The presence of *tcp*A, *tcp*I, *omp*U, and *zot* genes in some isolates, which are essential for colonisation, makes them potentially virulent. Even if *V. cholerae* strains were isolated constantly during the year, the potentially pathogenic strains were found during the summer months and soon after a human gastroenteritis outbreak. Besides virulence genes and the genetic elements mediating their transfer, the single most important contributor to the evolution of pathogenic *V. cholerae* is the human host itself, which supports the selective enrichment of pathogenic strains from a diverse mixture of environmental *Vibrio* strains. Pathogenic strains evolve from environmental forms that are able to colonise the intestine through the acquisition of new genetic elements by horizontal gene transfer (Bhowmick *et al.* 2009, Faruque *et al.* 2003, Li *et al.* 2014, Rivera *et al.* 2001).

The increase of population during the summer months in areas popular with tourists could create the appropriate environment for the development of strains that are more dangerous than resident bacterial flora.

A remarkable feature of all *Vibrio* species is the

highly plastic genome. The 2 chromosomes are shaped by horizontal gene transfer involving, among others, antibiotic resistance, virulence, and niche adaptation (Kirkup *et al.* 2010). The carriage of virulence factors by non-O1/non-O139 strains creates an environmental reservoir of critical virulence genes, which may contribute to the evolution of pathogenic *V. cholerae* through recombination events and horizontal gene transfer.

Further surveillance and investigation is required to understand the molecular evolution, epidemiology, and pathogenicity of non-O1/non-O139 isolates of environmental origin (Liu *et al.* 2014).

PFGE of 22 *V. cholerae* typeable strains gave 13 pulsotypes clustered in 3 groups. In spite of the use of thiourea, which is recommended to prevent electrophoresis-related DNA degradation (Zamudio *et al.* 2011), 8 (26.7%) *V. cholerae* strains could not be typed.

A linkage between cluster and season of sampling could be established since each cluster contained strains isolated during the same period. Typing results highlighted a variable genetic population clustering according to sampling period. Similarity between strains isolated during the summer period (cluster I) supports the point that environmental conditions could induce changes and acquisitions of genetic elements that create a 'sub-population' of potentially more pathogenic strains during the summer.

Many studies used PFGE analysis to compare clinical and environmental strains in order to establish epidemiological and phylogenetic relationships among strains from different countries and sources (Lizarraga *et al.* 2009, Staley *et al.* 2010, Tapchaisri *et al.* 2008, Teh *et al.* 2010, Zamudio *et al.* 2011). This could be useful for tracing epidemics of indigenous strains in order to improve environmental surveillance and carry-out a risk assessment at national or international levels.

In summary, our results highlighted a human health risk associated with the detection of potentially pathogenic *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in the waters of the Vibrata river, which are used for irrigation and are situated next to the swimming areas of Abruzzo coastline.

The continuous detection of potentially pathogenic non-O1/non-O139 *V. cholerae* strains underlined that a more comprehensive surveillance and investigation are required in order to understand their molecular evolution, epidemiology, and pathogenicity.

Surveillance at national level could have public-health implications, not only for tracing epidemics of existing strains, but also in order to recognise strains with new genotypes that may emerge in the future.

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