Occurrence of *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus* in the clam *Ruditapes philippinarum* (Adams & Reeve, 1850) from Emilia Romagna and Sardinia, Italy

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Abstract

Marine vibrios, *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholerae* are responsible of the majority of food-borne human infections by consumption of bivalve shellfish. The aim of the present study was to ascertain the occurrence of these bacteria, and their potential pathogenicity, in the Manila clam *R. philippinarum* from Emilia Romagna (ER) and Sardinia (SR) regions, Italy. Isolation was performed on CHROMagar™ vibrio with subculture on (thiosulfate-citrate-bile salts-sucrose) Agar and m-modified-cellobiose-polymyxin b-colistin (-CPC) Agar. Suspected strains were purified, biochemically characterized and genotyped by simplex polymerase chain reaction (PCR) for the specie-specific and pathogenic gene markers: *V. parahaemolyticus* (*toxR*P, *tdh* and *trh*); *V. vulnificus* (*vvhA*, *hsp*, *vcg*C, *vcg*E, CPS operon allele 1, CPS operon allele 2, 16s-rRNA operon allele A, 16s-rRNA operon allele B; *V. cholerae* (*toxR*C, *hlya*, *tcpI*, *tcpA*, *ctxA*, *ctxB*, *stn/sto*). Moreover a multiplex PCR was applied to the SR bivalve shellfish, for the simultaneous detection of the three targets directly on homogenate samples, targeting the species-specific gene for *V. cholerae* (*toxR*C), *V. parahaemolyticus* (*toxR*P) and *V. vulnificus* (*vvhA*). As a result of phenotyping and genotyping of isolates, bivalve shellfish from ER resulted positive for *V. parahaemolyticus* (27.8%) and *V. vulnificus* (10.1%), but negative for *V. cholerae*. Shellfish from SR resulted positive for *V. parahaemolyticus* (30.3%), *V. vulnificus* (6.1%) and *V. cholerae* (3%). No significant differences emerged between the two areas (P>0.05).

Introduction

Global total production of fish, crustaceans, molluscs and other aquatic animals has continued to increase in the last decades, as a result of a stable situation of total captures and a strong growth of aquaculture, particularly of bivalve shellfish, hereafter indicated simply shellfish, that reached a worldwide production of 14.8 million tons (FAO, 2014). The Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850) is one of the most cultivated species in the world, and Italy is the second producer after China (Turolla, 2008). The national production is concentrated in the North-west Adriatic sea, from Friuli-Venezia Giulia region to Emilia Romagna region, were *R. philippinarum* is cultured. In Sardinia region the production is actually marginal, and mainly by catch, but shows a promising trend for expansion.

As sedentary suspension feeding organisms, shellfish gain nourishment by pumping large volumes of seawater from the environment through their gills, and thus they may accumulate harmful bacteria and viruses resulting in a substantial health risk for the consumers, particularly if they are eaten raw or lightly cooked, as is often the case (Serratore *et al*., 2014). Focusing on bacterial contaminants, pathogenic vibrios are undoubtedly the most relevant ones from an epidemiological point of view. Members of the genus *Vibrio* are gram-negative straight or curved roads, ubiquitous in the marine environments. To date, one hundred and thirty species of vibrios have been described and thirteen have been classified as human pathogens (Thandavarayan *et al*., 2014), but with different epidemiological relevance. *V. parahaemolyticus, V. vulnificus and V. cholera* are considered a serious and growing public health hazard, whereas infections by the remaining vibrio species are less common and usually less severe, although deaths have been reported (European Commission, 2001).

V. cholerae is considered a foodborne pathogen that affects humans and causes cholera, a disease characterized by profuse watery diarrhea and vomiting, which can lead to severe dehydration, hypovolemic shock and, with no appropriate treatment, death. This disease is still a serious problem in some countries of Asia, African regions and Haiti and remains endemic in much of the developing world. In 1994, cholera outbreaks occurred also in Italy and Albania (Pazzani *et al*., 2006). Actually, more than 200 somatic (cell wall) O-antigen serogroups of *V. cholerae* are recognized, among which only serogroups O1 and O139 are considered etiologic agents of epidemic and pandemic cholera, but occasional outbreaks of cholera-like disease are also reported (Fykse *et al*., 2007). The pathogenicity of *V. cholerae* O1 and O139 depends upon the production of virulence factors, such as the toxin co-regulated pilus TCP and the cholera toxin CTX. Other factors associated with enteropathogenicity include: an El Tor-like hemolysin (hlyA), hemagglutinins, neuraminidase, outer membrane protein (ompU), Shiga-like toxin (Stx) and a zonula occludens toxin (Zot) (Rivera *et al*., 2001). The heat-stable enterotoxin named stn/sto often determines the pathogenicity of environmental non-O1 and non-O139 strains (Guglielmetti *et al*., 1994).

V. parahaemolyticus occurs naturally in the marine environments and may be abundant in shellfish, being recognized as a common cause of acute gastroenteritis worldwide (Ward and Bej, 2006). In Japan, *V. parahaemolyticus* accounts for approximately 20-30% of cases of bacterial foodborne infection, being the common cause of seafood-borne illness in many Asian countries, and has been recognized as

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Key words: *V. parahaemolyticus; V. cholerae; V. vulnificus; R. philippinarum;* Italy.

Contributions: PLP and EZ were involved in the isolation and phenotypical characterization of the bacterial targets. GB was involved in the genotyping of the isolates of interest. AS was involved in the revision of the paper. PS was involved in the analysis of literature, planning of the work, supervision of the laboratory procedures, and writing of the paper.

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the most frequent cause of *Vibrio*-associated gastroenteritis in the US (Ceccarelli *et al*, 2013).

V. parahaemolyticus gastroenteritis is related to the production of two hemolysin: a thermostable direct hemolysin (TDH) and/or a thermostable-related hemolysin (TRH) (Zhang and Austin, 2005). Infections caused by *V. parahaemolyticus* have been associated with diverse serovars, among which O1:K38, O3:K29, O4:K8, O3:K6, O2:K3, O4:K8, with a localized geographical distribution. Serovar O3:K6, showing specific gene markers (*tdh, toxRS/New* and *orf8*), may have a pandemic diffusion, (Velasquez-Roman *et al*., 2014). In Europe, *V. parahaemolyticus* infections have been rarely reported, but the O3:K6 serovar has been isolated from clinical specimens in France, Russia, Spain and Italy (Ottaviani *et al*., 2008).

V. vulnificus is considered an opportunistic human pathogen that is responsible for 95% of seafood-related deaths in the US (Williams *et al*., 2013). Actually, three biogroups are recognized and differentiated by biochemical characteristics: biogroup 1, which is pathogenic to humans, biogroup 2, which is pathogenic to eels, and biogroup 3, which is pathogenic to humans but has only been reported in Israel (Sanjuán *et al*., 2011). Biogroup 1 infects humans through the ingestion of contaminated seafood or skin lesions, and in healthy individuals may cause gastroenteritis or wound infection, whereas in immunocompromised hosts the infection often leads to primary or secondary septicemia, with a fatality rate exceeding 50 and 25%, respectively (Han *et al*., 2011).

For a long time, the identification of *Vibrio* spp. during epidemiological inquiries was mainly based on phenotypic markers for strains isolated at 37°C, but this approach is not reliable enough to identify strains collected from environmental samples, mainly because of the adaptation and subsequent phenotypic changes to varying environmental conditions (Tall *et al*., 2012). Moreover cultural methods utilized alone are incapable to distinguish pathogenic strains from non-pathogenic strains, thus molecular methods were developed to improve the detection and identifica-

Table 1. Biochemical characteristics of *Vibrio parahaemolyticus* strains isolated in the present study.

Strain	TCBS	KIA (lactose/sucrose/H_2S/gas)	A/L/O	Indole	Citrate	ONPG	Gelatinase	O/129 (150-10 μg)	Growth at 42°C
Emilia Romagna									
VP464/11	-	KA/-/-	-/+/+	+	+	-	+	SS	+
VP480/5	-	KA/+/-	-/+/+	+	+	-	+	SR	+
VP501/13	-	KA/-/-	-/+/+	+	+	-	+	SR	+
VP510/4	-	KA/-/-	-/+/-	+	+	-	-	SR	+
VP628/8	-	KA/+/-	-/+/+	+	+	-	+	SR	+
VP644/11	-	KA/-/-	-/+/+	+	-	-	+	SS	-
VP731-11	-	KA/-/-	-/+/+	+	+	-	+	SS	+
VP734-5	-	KA/-/-	-/+/+	+	+	-	+	SS	+
VP734-12	-	KA/-/-	-/+/+	+	+	-	-	SS	+
VP739-9	-	KA/-/-	-/+/-	+	+	-	+	SS	+
VP741-12	-	KA/-/-	-/+/+	+	+	+	+	SR	+
VP768-12	-	KA/-/-	-/+/+	+	+	-	+	SS	+
VP776-1	-	KA/-/-	-/+/-	+	+	-	+	SS	+
VP794-4	-	KA/-/-	-/+/-	+	+	-	+	SR	+
VP814-3	-	KA/-/-	-/+/+	+	-	-	+	SR	+
VP889-4	-	KA/-/-	-/+/-	+	-	-	+	SR	+
VP890-3	-	KA/-/-	-/+/-	+	-	-	+	SR	+
VP1022-2	-	KA/-/+	-/+/+	+	+	-	+	SR	+
VP1023-1	-	KA/-/-	-/+/+	+	+	-	+	SR	+
VP1031-2	-	KA/-/-	-/+/+	+	+	-	+	SR	+
VP1048-1	-	KA/-/-	-/+/+	+	+	-	+	SS	+
VP1048-6	+	KA/-/-	-/+/+	+	+	-	+	SS	+
VP1086-3	-	KA/-/-	-/+/+	+	+	-	+	SS	+
VP1087-6	-	KA/-/-	-/+/-	+	+	-	+	SS	+
Sardinia									
VP761-10	-	KA/-/-	-/+/-	+	+	-	+	SS	+
VP770-1	-	KA/-/-	-/+/-	+	+	-	+	SS	+
VP771-1	-	KA/-/-	-/+/-	+	+	+	+	SS	+
VP780-2	-	KA/-/-	-/+/-	+	+	-	+	SS	+
VP781-1	+	KA/-/-	-/+/-	+	+	-	+	SR	+
VP784-1	-	KA/-/-	-/+/+	+	+	-	+	SR	+
VP788-3	-	KA/-/-	-/+/-	+	+	-	+	SS	+
VP804-8	-	KA/-/+	-/+/+	+	-	+	+	SR	+
VP806-11	-	KA/-/+	-/+/-	+	-	-	+	SR	+
VP857-1	-	KA/-/-	-/+/+	+	+	-	+	SR	+

TCBS, thiosulfate-citrate-bile salts-sucrose; KIA, Kligler Iron Agar; ONPG, ortho-nitrophenyl- -galactoside; VP, Vibrio parahaemolyticus; KA, Kligler Agar; S, sensitive; R, resistant.

tion of culturable strains, mainly based on polymerase chain reaction (PCR) assays.

Vibrios seem to be highly variable in habitat preference (Schmidt *et al*., 2014); therefore prevalence studies are of paramount importance to define the risk of *Vibrio*-associated infectious diseases in a specific geographical area. The present study aims to give a contribute of knowledge on the prevalence and potential pathogenicity of *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* associated to the Manila clam *R. philippinarum* from two Italian regions, Emilia Romagna facing the Adriatic Sea, and Sardinia facing the Tyrrhenian Sea.

Materials and Methods

This study was performed by different year-long sampling campaigns, from 2011 to 2014. On the whole 112 batches of *R. philippinarum* were analyzed, 79 from Emilia Romagna (ER) and 33 from Sardinia (SR). Samples from ER were collected at landing and sent to the laboratory the same day, whereas those from SR were sent the day after landing. All samples were transported in isothermal boxes, stored at 6-8°C to avoid *Vibrio* spp. entering viable but non-culturable (VBNC) state, and processed within 24 h.

The abundance of *Vibrio* spp. was checked on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, Basingstoke, UK) NaCl 3% by the spread plate method, and incubation at 20°C for 3-5 days (Serratore *et al*., 1999). A significant number of colonies were tested to confirm the typical traits of the genus: Gram negative straight or curved rods, oxidase positive, able to reduce nitrate, dextrose fermenting, and sensitive to the vibriostatic O/129 (150 μg). The results were expressed as colony forming units (CFU) g^{-1}. The isolation of *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* was performed on CHROMagar™ Vibrio (CAV), following the triple plating methods described by Williams *et al*. (2013). Suspected colonies of V. *vulnificus* and *V. cholerae* (turquoise colour) and suspected colonies of *V. parahaemolyticus* (mauve colour), were transferred onto TCBS agar NaCl 3% and modified cellobiose-polymyxin B-colistin (m-CPC) agar, prepared according to the formulation of the Bacteriological Analytical Manual (Kaysner and DePaola, 2004). Presumptive *V. parahaemolyticus* strains (green on TCBS Agar, no growth on m-CPC Agar), presumptive *V. cholerae* strains (yellow on TCBS Agar, purple on m-CPC Agar), and presumptive *V. vulnificus* strains (green or yellow on TCBS Agar, yellow on m-CPC Agar), were purified on trypticase soy agar (TSA; Oxoid) NaCl 3%, tested for the genus-typical traits and then phenotypically characterized following the laboratory protocol, a slight modification of the scheme proposed by Alsina and Blanch as revised by Noguerola and Blanch (2008).

The strains tentatively confirmed to belong to the species of interest were genotyped by simplex PCR, following an internal protocol, for the specie-specific and pathogenic gene markers: *toxR*P, *tdh* and *trh* for *V. parahaemolyticus*; *toxR*C, *hlya*, *tcpI*, *tcpA*, *ctxA*, *ctxB*, *stn/sto* for *V. cholerae*; *vvhA*, *hsp*, *vcg*C, *vcg*E CPS operon allele 1, CPS operon allele 2, 16s-rRNA type *A* gene, 16s-rRNA type *B* gene for *V. vulnificus* (Serratore *et al*., 2009). Briefly, a small amount of the purified strain was picked from the plate and transferred in 100 µL of sterile H_2O. The DNA was extracted by the boiling method at 100°C for 15 min, stored 2-3 min at -20°C and then centrifuged at 5.000 x g at 4°C for 15 min, recovering the supernatant. Bacterial lysates were stored at -20°C until use. For the PCR reactions 1 μL of each lysate was suspended with 2.5 μL 10X PCR Rxn Buffer (Invitrogen, Carlsbad, CA, USA), 1 μL $MgSO_4$ (Invitrogen), 0.5 μL dNTPs 100 Mm (Invitrogen), 1 μL of each primer, 0.2 μL of Taq-DNA Polymerase (Invitrogen), and sterile H_2O to the mark of 25 μL. PCR reactions were electrophoresed at 100 V for 30 min on 1.5% agarose gel utilizing tris-acetate-eth-

Table 2. Biochemical characteristics of *Vibrio vulnificus* and *Vibrio cholerae* strains isolated in the present study.

Strain	Cellobiose (m-CPC)	TCBS	KIA	A/L/O	Indole	Citrate	ONPG	Gelatinase	O/129 (150-10 g)	Growth at 42°C
Emilia Romagna										
VV628-7	+	-	KA/-/-	-/+/+	+	-	+	+	SS	+
VV731-16	+	+	KA/-/-	-/+/-	+	-	+	+	SS	+
VV731-17	+	+	KA/-/-	-/+/+	+	-	+	-	SS	+
VV731-18	+	+	KA/-/-	-/+/+	+	+	+	+	SS	+
VV734-4	+	-	KA/-/-	-/+/-	+	-	+	-	SS	+
VV734-6	+	-	KA/-/-	-/+/-	+	-	+	+	SS	+
VV734-8	+	+	KA/-/-	-/+/+	+	-	+	-	SS	+
VV734-13	+	-	KA/-/-	-/+/-	+	+	+	+	SS	+
VV734-15	+	-	KA/-/-	-/+/-	+	-	+	+	SS	+
VV734-16	+	-	KA/-/-	-/+/-	+	-	+	+	SS	+
VV734-17	+	-	KA/-/-	-/+/-	+	-	+	-	SS	+
VV734-18	+	-	KA/-/-	-/+/-	+	-	+	-	SS	+
VV759-14	+	-	KA/-/-	-/+/-	+	+	+	+	SS	+
VV776-6	+	-	KA/-/-	-/+/-	+	+	+	+	SS	+
VV776-8	+	-	KA/-/-	-/+/+	+	-	+	+	SS	+
VV776-10	+	+	KA/-/-	-/+/+	+	-	+	+	SS	+
VV786-10	NG	-	KA/+/+	-/-/+	+	-	+	+	SS	+
VV1023-4	+	-	KA/-/-	-/+/-	+	-	+	+	SS	+
VV1023-5	+	-	KA/-/-	-/+/-	+	-	+	+	SS	+
VV1087-1	+	-	KA/-/-	-/+/-	+	-	+	-	SS	+
Sardinia										
VV770/11-3	+	-	KA/-/-	-/+/+	+	+	+	+	SS	+
VV770/11-4	+	-	KA/-/-	-/+/+	+	-	+	+	SS	+
VV780/11-4	+	-	KA/-/-	-/+/-	+	-	+	+	SS	+
VC780-6	-	+	KA/-/-	-/+/+	+	-	+	+	SS	+

CPC, cellobiose-polymyxin b-colistin; TCBS, thiosulfate-citrate-bile salts-sucrose; KIA, Kligler Iron Agar; ONPG, ortho-nitrophenyl-β-galactoside; VV, *Vibrio vulnificus*; KA, Kligler Agar; S, sensitive; R, resistant; NG, no growth; VC, *Vibrio cholerae.*

ylenediaminetetraacetic acid (TAE) buffer (Sigma Aldrich, St. Louis, MO, USA); Gel Red Nucleic Acid Stain (Biotium, Hayward, CA, USA) was utilized as fluorescent nucleic acid stain, and bromophenol blue with 30% glycerol in PCR water and TrackIt™ 100 bp DNA Ladder (Invitrogen) as loading buffer. The PCR products were evidenced by the transilluminator Bio-Rad Gel Doc™ XR, Trans-UV at 302 nm.

Samples from SR were also analyzed utilizing a multiplex PCR protocol developed in our laboratory for the simultaneous detection of *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* directly on homogenate samples, targeting the species-specific genes for *V. cholerae* (*toxR*C), *V. parahaemolyticus* (*toxR*P) and *V. vulnificus* (*vvhA*). Briefly, 25 g of shellfish homogenate was enriched overnight in 225 mL of Alkaline Peptone Water (Oxoid), then 1 mL was centrifuged at 19.000 x g at 4°C for 5 min. After the removal of supernatant, the pellet was washed with 1 mL of sterile H_2O, and the PCR reaction was performed following the same steps already described for the simplex PCR. To compare data obtained from shellfish of the two investigated areas, statistical analyses were performed by the unpaired t-test for *Vibrio* spp. abundance, and the chi-square test for the percentage of detection of *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*, utilizing the Prism version 6.0 software (GraphPad Software Inc, San Diego, CA, USA). A confidence interval at the 95% level ($P<0.05$) was considered significant.

Results

In order to be concise, single data on *Vibrio* spp. abundance are omitted. The mean value of *Vibrio* spp., expressed as colony-forming units (CFU g^{-1}) resulted 4.74 Log_{10} (SD=0.66) in shellfish from ER and 5.34 Log_{10} (SD=1.36) in shellfish from SR, pointing at a significant difference between the two areas ($P<0.05$).

Results on the isolation and characterization of *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* are reported taking into consideration more than one strain for samples only when isolates showed different genotype or phenotype. The biochemical characteristics of isolates confirmed as *V. parahaemolyticus*, 24 from ER samples and 10 from SR samples, are reported in Table 1.

Two samples from ER harbored simultaneously different strains (VP734 strains 5 and 12; VP1048 strains 1 and 6), and a discrete number of strains showed unusual characteristics, being sucrose positive on TCBS (5.9%), producing gas or H_2S on Kligler Iron Agar (KIA) (14.7%), ornithine negative (32.4%), sensitive to O/129 10 μg (50%), ortho-nitrophenyl-β-galactoside (ONPG) positive (8.8%), citrate negative (26.1%), gelatinase negative (26.1%). The biochemical characteristics of isolates confirmed as *V. vulnificus*, 20 from ER and 3 from SR, are reported in Table 2.

Different strains were simultaneously present in 3 samples from ER (VV731 strains 16, 17, 18; VV734 strains 4, 6, 8; VV776 strains 6, 8, 10) and 1 samples from SR (VV770/11 strains 3 and 4), and a discrete number of strains showed unusual characteristics, being sucrose positive on TCBS (26.1%), no growth on m-CPC agar (4.3%) producing gas and H_2S on KIA (4.3%), ornithine negative (56.5%), lysine negative (4.3%), citrate positive (26.1%),

Table 3. Polymerase chain reaction-based genotyping of *Vibrio vulnificus* and *Vibrio cholerae* strains isolated in the present study.

Strain	*vvhA*	*hsp*	*vcgC*	16S B	CPS1	*vcgE*	16S A	CPS2
Emilia Romagna								
VV628-7	+	+	-	-	+	+	+	-
VV731-16	+	+	-	-	-	+	+	+
VV731-17	+	+	-	-	-	+	+	+
VV731-18	+	+	-	-	-	+	+	+
VV734-4	+	+	+	+	+	-	-	-
VV734-6	+	+	-	-	-	+	+	+
VV734-8	+	+	-	-	+	+	+	-
VV734-13	+	+	-	-	-	+	+	+
VV734-15	+	+	-	-	-	+	+	+
VV734-16	+	+	+	+	+	-	-	-
VV734-17	+	+	+	+	+	-	-	-
VV734-18	+	+	-	-	-	+	+	+
VV759-14	+	+	-	-	-	+	+	+
VV776-6	+	+	-	-	-	+	+	+
VV776-8	+	+	-	-	-	+	+	+
VV776-10	+	+	-	-	-	+	+	-
VV786-10	+	+	+	+	+	-	-	-
VV1023-4	+	+	-	-	+	+	+	-
VV1023-5	+	+	+	-	+	+	+	+
VV1087-1	+	+	-	-	+	+	+	+
Sardinia								
VV770/11-3	+	+	-	-	-	+	+	+
VV770/11-4	+	+	-	-	-	+	+	+
VV780/11-4	+	+	-	-	-	+	+	-
	toxRC	*hlyA*	*tcpI*	*tcpA*	*ctxA*	*ctxB*	*stn/sto*	
VC780-6	+	+	-	-	-	-	-	

VV, *Vibrio vulnificus;* VC, *Vibrio cholerae.*

gelatinase negative (26.1%). The only one strain confirmed as *V. cholerae* (Table 2) showed the typical phenotypic traits of the specie, being sucrose positive on TCBS, gas and H_2S not produced on KIA, arginine negative, lysine and ornithine positive, sensitive to O/129 10 μg, ONPG positive, citrate negative.

The genotyping of *V. parahaemolyticus* was performed for 3 gene markers, and considering redundant the presentation of a table, data are omitted. All strains showed positivity to the specie-specific gene marker *toxR*P. Among the isolates from ER shellfish, 27.3% were positive for the pathogenic gene markers, of which 18.2% *trh* positive (VP731/11, VP734/12, VP1048/1, VP1087/6), and 9.1% *tdh* positive (VP734-5 e VP1048-6), with samples VP734 and VP1048 harboring simultaneously different strains. None of the isolates from SR shellfish showed positivity to the pathogenic gene markers. The genotyping of the *V. vulnificus* isolates, performed by several gene markers, justifies a complete presentation, and data are reported in Table 3. All isolates were positive to the specie-specific gene markers *vvha* and *hsp* and those from ER shellfish evidenced 37.5% positivity for the *vcg*C gene.

The complete environmental pattern (*vcg*E, 16S A, CPS2) was found in 56.5% of the strains, and the complete clinical pattern (*vcg*C, 16S B, CPS1) in 17.4% of the strains, all from ER samples. The remaining strains showed a mixed pattern being *vcg*E, 16S A, CPS1 (17.4%) or untypeable for CPS marker, being neither CPS1 nor CPS2 positive (8.7%). As a result of phenotyping and genotyping of isolates, shellfish batches from ER resulted positive for *V. parahaemolyticus* (27.8%) and *V. vulnificus* (10.1%), but negative for *V. cholerae*. Shellfish batches from SR resulted positive for *V. parahaemolyticus* (30.3%), *V. vulnificus* (6.1%) and *V. cholerae* (3%). The only one strain of *V. cholerae* isolated from SR shellfish was negative to all the pathogenic gene markers utilized in the study (Table 3). No significant differences emerged between the two areas ($P>0.05$).

The application of our Multiplex PCR protocol, performed on SR samples, confirmed the results obtained by the cultural method for *V. vulnificus*, while evidenced more than twofold the positivity for *V. parahaemolyticus* (63.6 *vs* 30.3%). These data suggest that *V. parahaemolyticus* population in shellfish may comprise a consistent amount of dead and/or viable but not culturable strains. The failure to evidence *V. cholerae* was unexpected, and requires further investigations.

Discussion

Members of the genus *Vibrio* are ubiquitous heterotrophic bacteria of the marine environments, and are normally accumulated in shellfish, representing a feed source together with plankton and other organisms. The present study focused on *Vibrio* spp. abundance and the prevalence of the potentially pathogenic members of the genus *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in *R. philippinarum* from two Italian regions, ER and SR. Notwithstanding the high ecological diversity between ER and SR marine production areas, only the difference on the abundance of *Vibrio* spp., being 4.74 Log_{10} and 5.34 Log_{10} respectively, resulted statistically significant ($P<0.05$), whereas the prevalence of samples positive for the target species, was not statistically significant ($P>0.05$). This fact suggests that the presence of the potentially pathogenic subpopulations of environmental vibrios is unrelated to the total vibrio population, as reported elsewhere (Johnson *et al*., 2012).

The utilization of TCBS agar NaCl 3% with incubation at 20°C, allowed to the recovery of *Vibrio* spp. in all samples, with an abundance indubitably higher than previously reported in environmental samples from the Adriatic Sea, where TCBS agar without NaCl added and/or incubation at 37°C, were employed (Barbieri *et al*., 1999; Ripabelli *et al*., 1999; Rubini *et al*., 2009).

The triple plating methods proposed by Williams *et al*. (2013) for *V. vulnificus*, allowed to the recovery of sucrose positive variants of *V. parahaemolyticus* and *V. vulnificus,* and *V. vulnificus* variants unable to grow on m-CPC agar, confirming that CAV easily differentiates *V. parahaemolyticus* from *V. vulnificus*, *V. cholerae* and other vibrios with great sensitivity, as reported elsewhere (Di Pinto *et al*., 2011).

The prevalence of *V. parahaemolyticus* in ER shellfish resulted 27.8%, in agreement with another study on shellfish from Veneto region, facing the North Adriatic Sea as well, showing a positivity of 27.5%. In the same study, the prevalence of *V. parahaemolyticus* in shellfish from Sardinia resulted 56.7% (Suffredini *et al*., 2014), significantly higher with respect to our study, where the positivity of SR shellfish resulted 30.3%.

Potentially pathogenic *V. parahaemolyticus* strains were recovered only from ER shellfish with a percentage of 27.3%, and similar values (33.3%) have been reported for strains isolated from mussels of the Ionian Sea, even if targeting only the *tdh* gene (Di Pinto *et al*., 2008), whereas our strains resulted 18% *trh* + and 9.1% *tdh* +.

V. cholerae was recovered in 3% of SR shellfish sample and was absent in ER shellfish samples. These low values of prevalence are in agreement with data previously reported for Italian mussels (Ottaviani *et al*., 2009). *V. vulnificus* was found in 10.1% of ER shellfish and 6.1% of SR shellfish, but scant information are available for a comparison.

The phenotypic and genetic traits of *V. vulnificus* strains isolated in the present study were highly variable, as previously reviewed (Jones and Oliver, 2009), whereas the variability of *V. parahaemolyticus* resulted higher than commonly accepted, but confirming some recent findings (Rojas *et al*., 2011).

Conclusions

The popularity of shellfish in the diet is growing, however, concerns have been raised worldwide regarding health risks from shellfish contaminated with human pathogens of anthropogenic origin, particularly the enteroviruses, and the autochthonous bacteria *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*. Moreover, it is useful to outline that the depuration treatment, utilized worldwide to purge shellfish from faecal contaminants in clean seawater, is largely considered unsatisfactory with respect to enteroviruses and vibrios (Serratore *et al*., 2014). In order to develop measures for human health protection based on a robust risk analysis, the acquisition of data on the prevalence of *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*, and their potential pathogenicity traits, is fundamental. On the other hand, the relatively low prevalence of potentially pathogenic strains emerged in the present study, even if not negligible, outline the necessity to couple cultural methods with the genotyping approach, to properly identify safe versus unsafe shellfish, avoiding unjustified economical losses consequent to the rejection of product evaluated only on the basis of the cultural methods, that may give false positive results and are unable to ascertain the potential pathogenicity of isolates. Giving the scant information of concern actually available for the Italian production areas, further researches are needed, but above all, the competent authorities should become aware of the necessity to implement a systematic microbiological control of shellfish production areas, today routinely limited to the faecal contaminants, including an appropriate survey of *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*.

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