RESEARCH LETTER

Detection of pathogenic *Vibrio parahaemolyticus* through biochemical and molecular-based methodologies in coastal waters of the Gulf of Trieste (North Adriatic Sea)

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Abstract

Culturable vibrios were isolated from seawater collected during an annual sampling study performed along the Gulf of Trieste coast (Northern Adriatic Sea), and conventional culturing and identification methods were used to investigate the presence of *Vibrio parahaemolyticus*. Biochemically selected *Vibrio* strains were subjected to phenotypical identification performed using Alsina's scheme, API 20E and API 20NE. PCR and sequence analysis of the 16S rRNA gene and detection of the species-specific *toxR* and *tlh* genes were carried out on strains presumptively identified as *V. parahaemolyticus* and on a set of unidentified strains to confirm biochemical characterizations. In addition, PCR assays targeting the virulence genes, *tdh* and *trh*, were carried out to detect pathogenic strains. PCR results were compared with phenotypic characterizations to evaluate the accuracy of the biochemical methods applied. False-negative identifications were obtained by all phenotypic-based procedures, while API 20E yielded only one false positive. Because the amplification of the 16S rRNA gene produced uncertain results, *toxR* and *tlh* gene detections were necessary to confirm the biochemical identifications. Finally, molecular characterization demonstrated the presence of *V. parahaemolyticus trh*-positive strains and underlined the difficulty in the recognition of the pathogenic environmental organism using conventional methods.

Introduction

Vibrio parahaemolyticus is a marine bacterium easily recovered from estuarine and coastal waters worldwide (Kaneko & Colwell, 1975; Joseph *et al.*, 1982; Karunasagar *et al.*, 1987; DePaola *et al.*, 1990). As well as from seawater, it has been isolated from sediment, suspended particles (Colwell, 1984) and from a wide variety of marine organisms (Drake *et al.*, 2007 and references therein), such as crustaceans (Kaneko & Colwell, 1975; Wong *et al.*, 1999) and molluscs (DePaola *et al.*, 1990; Croci *et al.*, 2001; DePaola *et al.*, 2003a, b; Ottaviani *et al.*, 2005). Food-borne infections caused by this organism usually present as gastroenteritis exclusively associated with the consumption of raw or improperly cooked contaminated fish and shellfish; *V. parahaemolyticus* can cause skin infections by contact of an open wound with seawater (Daniels *et al.*, 2000). *Vibrio parahaemolyticus* is well known as an important human pathogen (Thompson *et al.*, 2004 and references therein; Ottaviani *et al.*, 2005 and references therein), especially in some Asian countries (Joseph *et al.*, 1982) and in the United States (Daniels *et al.*, 2000). Recently, cases of infections were also reported in Europe (Martinez-Urtaza *et al.*, 2004; Ottaviani *et al.*, 2008 and references therein). In Italy, the first report on the clinical isolation of a pandemic *V. parahaemolyticus* strain, with local shellfish as the most probable source of the infection (Ottaviani *et al.*, 2008), and previous investigations that showed the presence of pathogenic *V. parahaemolyticus* in the Adriatic Sea environment (Ottaviani *et al.*, 2005; Caburlotto *et al.*, 2008) have created renewed interest in the spread of pathogenic traits along Italian coastal areas. Italian laboratories performing official controls utilize conventional cultural methods to recover and identify the environmental vibrios, but the application of biochemical-based procedures often does not produce reliable results (Austin *et al.*, 1997; Croci *et al.*, 2007). However,

due to the presence of both false-positive and false-negative results in all the biochemical identification methods proposed, some authors (O'Hara *et al.*, 2003; Thompson *et al.*, 2004; Croci *et al.*, 2007) suggested caution in the interpretation of such identifications and advise the use of additional confirmatory testing, such as PCR, which enables the detection of the specific nucleotide sequence of *V. parahaemolyticus*. To specifically detect *V. parahaemolyticus* by PCR, several researchers used the species-specific targets *toxR* gene (Kim *et al.*, 1999; Deepanjali *et al.*, 2005; Croci *et al.*, 2007) and the thermolabile hemolysin gene (*tlh*) (Bej *et al.*, 1999). Recently Croci *et al.* (2007), utilizing *Vibrio* strains (reference, environmental and clinical strains) already identified by API 20E, API 20NE (API; bioMérieux, Marcy l'Etoile, France) and Alsina's scheme (Alsina & Blanch 1994a, b), conducted a multicenter evaluation of biochemical and molecular methods for *V. parahaemolyticus* identification and found that Alsina's scheme for biochemical characterization and *toxR* gene detection for molecular analyses produced the best results for inclusivity, exclusivity and concordance. In addition, to determine the real risk posed to human health by the presence of *V. parahaemolyticus*, strain identifications must be followed by the detection of the pathogenicity marker genes: *tdh* (thermostable-direct hemolysin) and *trh* (thermostable-related hemolysin) (Bej *et al.*, 1999).

In the present study, aimed at investigating the presence of *V. parahaemolyticus* in two coastal sites in the Gulf of Trieste (North Adriatic Sea), to select environmental strains, we used the same three biochemical identification methods (Alsina's scheme, API 20E and API 20NE) using media and bacterial suspensions with a slight modification of the salinity from 0.9% to 3% NaCl. Subsequent molecular analyses were performed to confirm phenotypic characterizations. The PCR results for the 16S rRNA gene, *toxR* and *tlh* genes were compared with biochemical characterizations of *V. parahaemolyticus* environmental strains to evaluate the effectiveness of the biochemical methods applied. Finally, to investigate the spreading of pathogenic traits, the isolates were subjected to PCR assays to detect *tdh* and *trh* genes.

Materials and methods

Study area and sampling

The environmental strains had been isolated from a total of 24 seawater samples collected during a monitoring program carried out monthly throughout 2003, which aimed to investigate the presence of vibrios in two sites in the Gulf of Trieste (NE Adriatic Sea): C1 (45°42′03″N, 813°42′36″E) is about 200 m offshore and D2 (45°45′49″N, 13°35′36″E) is 1250 m offshore and is located near the Isonzo River delta.

Surface (−0.5 m) water samples were collected with a 10-L Niskin bottle, kept in 2-L polyethylene bottles (washed with 10% HCl and rinsed in MilliQ water), stored in freezing bags (6 ± 2 °C) and processed within 2 h after collection.

Isolation of the strains

The recovery of vibrios from seawater was performed using conventional cultural methods (Elliot *et al.*, 2001), optimally adapted to water samples: seawater (1 L) was filtered through 0.22-μm-pore-size polycarbonate membranes and then incubated in alkaline peptone water at 36 ± 1 °C; after 24 h, a loopful of enrichment broth was streaked onto thiosulfate–citrate–bile–sucrose (TCBS) agar and then maintained at 37 °C for 24 h. Preliminary identification of the strains had been performed on the basis of colony morphology and sucrose utilization on TCBS. Sucrose-negative (*sac*–) strains were cultured on 3% NaCl tryptone soy agar (TSA, Oxoid, Basingstoke, UK) and stored at 10 °C in 3% NaCl TSA tubes overlaid with mineral oil.

Two *V. parahaemolyticus* reference strains were selected from international collections (ATCC 43996 and ATCC 17802 – American Type Culture Collection, Manassas, VA) and were utilized in biochemical and molecular analyses. In particular, we utilized ATCC 43996 ($toxR^+/tlh^+/tdh^+$) and ATCC 17802 ($toxR^+/tlh^+/trh^+$) as PCR-positive controls (Yang *et al.*, 2008) and distilled water as a negative control. Each molecular analysis was performed in triplicate.

Biochemical methods

Phenotypic identifications were performed using the following three steps: to confirm the typical traits of the *Vibrio* genus (screening phase), the strains cultured on 3% NaCl TSA (36 ± 1 °C) were subjected to a set of six tests (Gram staining, oxidase test, fermentative degradation of dextrose, nitrate reduction, motility test and growth under anaerobic conditions) (Elliot *et al.*, 2001); all the biochemical media were prepared including 3% NaCl. The fermentative degradation of dextrose was tested on ZOF medium: Marine ZoBell 0.3% agar at pH 7.6 ± 0.2, with 0.01% phenol red and 1% dextrose added after sterilization (Lemos *et al.*, 1985). For growth under anaerobic conditions, storage responses were considered. In the second phase, bacterial strains confirmed as *Vibrio* were subjected to the following tests referred by Elliot *et al.* (2001), with the exception of salt tolerance in 0/6/8% and 12% NaCl tryptone water (Baumann & Baumann, 1981): growth at 42 °C, the arginine dihydrolase test, O/129 Vibriostat sensitivity (10 and 150 μg) (bioMérieux) and Kliger Iron agar test. Finally, the strains presumptively identified as *V. parahaemolyticus* were subjected to biochemical identification using commercially available miniaturized systems API 20E and API 20NE

(bioMérieux). The bacterial suspensions were prepared in 7 mL of a 3% NaCl solution instead of the recommended 0.85% NaCl medium. The incubation time and temperature were maintained within the limits prescribed by the supplier (for API 20E 37 ± 1 °C for 24 h, for API 20NE 30 ± 1 °C for 24+24 h). Identifications were carried out using the APILAB PLUS 3.3.3 software (bioMérieux), and were considered acceptable on yielding a probability ≥80%.

Biochemical identifications were also performed using Alsina's scheme (Alsina & Blanch, 1994a, b), optimized by Ottaviani *et al.* (2003), based on biochemical tests grouped into identification keys. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, acetoin production, *N*-acetyl-glucosamine assimilation, utilization of citrate and D-glucosamine responses were recorded from API strips. In addition, some indications from the *Bergey*'s *Manual of Determinative Bacteriology* (Holt *et al.*, 1994) about assimilation activity, such as for capric acid and amygdaline, were considered. Because of the extension of the identification scheme, only the identification of *V. parahaemolyticus* strains was followed.

Molecular methods

The biochemically identified *V. parahaemolyticus* strains were cultured in 3% NaCl tryptone soy broth (Oxoid), at 37 ± 1 °C for 24 h, to confirm their identities by (1) PCR amplification and sequencing of the 16S rRNA gene and (2) PCR amplification to detect the presence of the *toxR* (Kim *et al.*, 1999), *tlh* (Bej *et al.*, 1999), *tdh* and *trh* genes (Bej *et al.*, 1999). Nucleic acid extraction was performed using the DNeasy™ Tissue Kit, Qiagen, according to the manufacturer's instructions. Briefly, bacterial cultures (1.5 mL) were centrifuged at 6000 ***g*** for 10 min and pellets were resuspended in a lysis buffer, then 20 μL of Proteinase K was added and the solution was incubated at 55 °C for 2 h. Then we added 200 μL of a buffer solution and the samples were incubated at 70 °C for 10 min. Finally, we added 200 μL of ethanol (96%), and after two centrifugations at 6800 ***g*** for 1 min, the DNA extracted was ready for PCR amplification. The extract was quantified fluorometrically (Perkin Elmer LS50B) using the PicoGreen dsDNA quantitation kit (Molecular Probes).

A portion of the 16S rRNA gene was amplified by a modification of the touchdown protocol (Don *et al.*, 1991) using the universal primer 27F and the eubacterial-specific primer 1492R (Lane, 1991). An initial 94 °C denaturing step for 5 min was followed by 30 cycles of amplification (3-min denaturation at 94 °C; 1-min annealing starting at 65 °C for the first cycle reduced from 0.5 °C per cycle to 50 °C; 3-min extension at 72 °C), five additional cycles of amplification (3 min at 94 °C; 1 min at 50 °C; 3 min at 72 °C) and a final extension of 10 min at 72 °C.

The detection of the *toxR*, *tlh*, *tdh* and *trh* genes was performed according to Kim *et al.* (1999) and Bej *et al.* (1999). For each amplification, the following reaction mixture was used: 1 μL of the template, 5 μL of 10 × HotMaster Taq Buffer with Mg^{2+} (Eppendorf), 5 μL of each primer (10 μM) (Sigma-Genosys Ltd), 1 μL of deoxynucleoside triphosphates (10 mM), 0.4 μL of *Taq* polymerase and H_2O to a final volume of 50 μL. The PCR products from five different amplifications were electrophoresed on 0.8% agarose gels and stained with ethidium bromide (0.5 μg mL^{-1}) and we used the DirectLoad™ Step Ladder (Sigma-Genosys Ltd) molecular weight marker to confirm amplification of the DNA fragments (368 bp for *toxR*, 450 bp for *tlh*, 269 for *tdh*, 500 bp for *trh* and almost 1400 bp for the 16S rRNA gene were the expected product sizes). PCR products were purified using the UltraClean™ PCR Clean-up Kit (MoBio) according to the manufacturer's instructions. 16S rRNA gene nucleotide sequences were determined using ABI Prism® BigDye™ dye-terminator chemistry (Applied Biosystems) and an automated ABI Prism® 3700 Genetic Analyzer (Applied Biosystems). Sequences were aligned to known sequences in the GenBank database using BLAST (Altschul *et al.*, 1990). To identify possible chimeras within the 16S sequences, all sequences were analyzed using the RDP program CHECK_CHIMERA. The sequences obtained in this study were deposited in the GenBank database under accession numbers GQ332269–GQ332300.

Statistical analyses

The effectiveness, of each biochemical method and for a group of tests, was evaluated based on sensitivity and specificity. One hundred percent sensitivity was sought in order to eliminate false negatives. Sensitivity and specificity were calculated as follows: sensitivity = [(number of isolates positive as determined by biochemical tests and PCR)/(total number of isolates positive as determined by PCR)] × 100; specificity = [(number of isolates negative as determined by biochemical tests and PCR)/(total number of isolates negative as determined by PCR)] × 100 (Choopun *et al.*, 2002).

Results

The environmental conditions in both sampling sites are well described in Celussi & Cataletto (2007): seawater temperature ranged from 6.4 to 25.3 °C following a typical seasonal progression, while the salinity showed a different trend: in C1, it ranged between 37.0 and 38.2 p.s.u., remaining fairly constant throughout the year, while in D2, we detected strong variations underlined by a wide annual range between 25.5 and 37.7 p.s.u. D2 is, in fact, located more close to the Isonzo River mouth and the season-dependent amount of freshwater inputs is reflected in strong variations in salinity.

Out of the 269 sucrose-negative isolates subjected to the screening phase, only 171 were confirmed as *Vibrio* spp. and then analyzed to verify their identity as *V. parahaemolyticus*. Twenty-three strains died during the analyses; 35 strains showed an arginine dihydrolase-positive reaction that is inconsistent with a *V. parahaemolyticus* typical response. One hundred and thirteen strains selected as presumptive *V. parahaemolyticus* were tested using API systems, and even among these, three strains yielded K/K in the KIA test, 32 strains were sensitive to 10 μg Vibriostat O/129 and 40 did not grow in 8% NaCl. API systems characterized only 19 strains as *V. parahaemolyticus* (Table 1); the urease production was recorded only for one strain (#PVP408).

PCR amplification and sequencing of the 16S rRNA gene and the detection of *toxR*, *tlh*, *tdh* and *trh* genes were carried out on 32 strains (19 presumptively identified as *V. parahaemolyticus* and 13 unidentified strains); the results for molecular analyses are summarized in Table 1. PCR 16S rRNA gene analyses identified 18 strains as *V. parahaemolyticus* with 100% identity, but yielded uncertain identification for 14 isolates. Twenty-one strains were confirmed as *V. parahaemolyticus* by PCR assays to detect species-specific targets (in Fig. 1 an example of ToxR PCR detection is shown); three strains were *trh* positive.

The comparison of biochemical and molecular results (Table 1) showed that, among the 21 *V. parahaemolyticus* strains, 19 were identified by one or both API systems, but only two of them yielded coherent responses with biochemical features reported by Alsina's scheme; in particular, API 20E yielded only one false positive (Table 2) and six false

Table 1. Results for each biochemical and molecular method performed

Strains	API 20E	API 20NE	Alsina's scheme	16S rRNA gene analyses	*toxR* gene	*tlh* gene	*tdh* gene	*trh* gene
ATCC 17802	−	+	−	*V. parahaemolyticus* 100%	+	+	−	+
ATCC 43996	+	−	−	*V. parahaemolyticus* 100%	+	+	+	−
PVP67	+	−	−	*V. parahaemolyticus/V. alginolyticus* 98%	−	−	−	−
PVP174	−	+	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP176	+	+	−	*V. parahaemolyticus/V. alginolyticus* 99%	+	+	−	+
PVP179	−	+	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP282	+	−	−	*V. parahaemolyticus* 99%	+	+	−	−
PVP326	+	+	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP357	+	+	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP358	+	+	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP359	+	−	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP360	+	+	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP378	+	+	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP379	+	+	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP380	+	−	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP387	+	−	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP399	+	−	−	*V. parahaemolyticus* 100%	+	+	−	−
PVP403	+	−	−	*V. parahaemolyticus* 100%	+	+	−	+
PVP407	−	+	+	*V. parahaemolyticus* 100%	+	+	−	−
PVP408	+	+	−	*V. parahaemolyticus* 100%	+	+	−	+
PVP411	−	+	+	*V. parahaemolyticus* 100%	+	+	−	−
NVP61	−	−	−	*V. parahaemolyticus/V. alginolyticus* 100%	−	−	−	−
NVP66	−	−	−	*V. parahaemolyticus/V. alginolyticus* 98%	−	−	−	−
NVP74	−	−	−	*V. parahaemolyticus/V. alginolyticus* 99%	+	+	−	−
NVP90	−	−	−	*V. parahaemolyticus/V. alginolyticus* 100%	−	−	−	−
NVP96	−	−	−	*V. parahaemolyticus/V. alginolyticus* 100%	−	−	−	−
NVP146	−	−	−	*V. parahaemolyticus/V. alginolyticus* 99%	−	−	−	−
NVP155	−	−	−	*V. parahaemolyticus/V. alginolyticus* 100%	−	−	−	−
NVP158	−	−	−	*V. parahaemolyticus/V. alginolyticus* 100%	−	−	−	−
NVP169	−	−	−	*V. parahaemolyticus/V. alginolyticus* 99%	−	−	−	−
NVP175	−	−	−	*V. parahaemolyticus/V. alginolyticus* 99%	−	−	−	−
NVP303	−	−	−	*V. parahaemolyticus* 100%	+	+	−	−
NVP395	−	−	−	*V. parahaemolyticus* 100%	+	+	−	−
NVP424	−	−	−	*V. parahaemolyticus/V. alginolyticus* 100%	−	−	−	−

+, a positive reaction or the presence of a PCR product; −, a negative reaction or no PCR product.

16S rRNA gene sequences of some strains (NVP61, NVP66, NVP74 and others) aligned to known sequences in the GenBank database using BLAST showed an unclear identification (*Vibrio parahaemolyticus/Vibrio alginolyticus*) with different percentages of similarity because the two species are phylogenetically very close.

negatives, while API 20NE yielded no false-positive results, but eight false negatives.

Discussion

The results obtained in the present work contribute to the debate about the problematic phenotypic identification of environmental *V. parahaemolyticus* strains. TCBS agar is the only proven selective medium for *Vibrio* spp. isolation, but a large number of marine microorganisms may also grow (Thompson *et al.*, 2004). In this study, the screening phase selected 58% of the analyzed strains as belonging to genus *Vibrio*. Our results confirm those of Croci *et al.* (2001), who evidenced how strains isolated from seawater and mussels on TCBS agar were principally vibrios (about 50%) while the remaining were *Aeromonas*, *Pseudomonas*, *Flavobacterium*, *Pasteurella* and *Agrobacterium*.

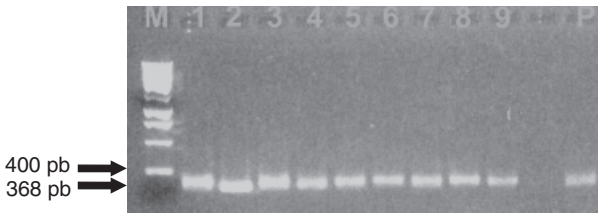

Fig. 1. Gel electrophoresis on an agarose gel of *toxR* PCR products. M = molecular weight marker DNA Ladder (Sigma-Genosys Ltd), 1–9 = bacterial isolates identified as *Vibrio parahaemolyticus*, P = positive control (*V. parahaemolyticus* ATCC 17802). Some molecular size markers are given on the left.

Table 2. Correspondences with the intended result and accuracy (sensitivity and specificity) for the biochemical methods performed

	API 20E	API 20NE	Alsina's scheme
Positive results	14 (21)	12 (21)	2 (21)
Negative results	10 (11)	11 (11)	11 (11)
False negative	7	9	19
False positive	1	0	0
Sensitivity %	67	57	9
Specificity %	91	100	100

API systems and Alsina's scheme (Alsina & Blanch, 1994a, b) are the most extensively used techniques by Italian Laboratories to screen the diversity of *Vibrio* spp. strains associated with marine organisms and their habitats (Croci *et al.*, 2007). However, several authors reported that *V. parahaemolyticus* phenotypic identification is difficult because of the huge variability of diagnostic features among the species (O'Hara *et al.*, 2003; Thompson *et al.*, 2004 and references therein; Croci *et al.*, 2007) and the molecular analyses considered necessary, either for additional confirmatory testing or for a certain identification method. In our study, the amplification of the 16S rRNA gene produced misidentifications because of the strictly genetic similarity between *V. parahaemolyticus* and *Vibrio alginolyticus*, *Vibrio campbelli*, *Vibrio carchariae* and *Vibrio harveyi* (Dorsch *et al.*, 1992). Molecular confirmation performed through PCR assays for *toxR* and *tlh* genes produced the same results in contrast to that reported by Croci *et al.* (2007), who reported that *tlh* gene detection yields false-positive identifications.

Although different studies highlighted the inadequacy of API systems for *Vibrio* identification (Dalsgaard *et al.*, 1996; Colodner *et al.*, 2004; Croci *et al.*, 2007), in the research, the use of both API 20E and API 20NE, using bacterial suspensions with a slight modification of the salinity from 0.9% to 3% NaCl, showed good results (Table 2); API systems displayed a higher sensitivity in comparison with Alsina's scheme, as reported by other authors (Toti *et al.*, 1996; O'Hara *et al.*, 2003), because the dichotomous method only identifies isolates with metabolic profiles strictly coherent with those reported by identification keys.

The majority of the molecular analyses confirmed that *V. parahaemolyticus* strains were not adherent with the phenotypic traits of the species that are considered diagnostic (Table 3 – false negative); assimilation activity for capric acid and amygdaline showed a huge variability among the selected strains, as reported by *Bergey*'s *Manual of Determinative Bacteriology* (Holt *et al.*, 1994), and was not useful as a diagnostic trait. The sensitivity and specificity evaluated for this group of biochemical tests were low (Table 3), in particular for resistance to Vibriostatic O/129 (10 μg) and

Table 3. Correspondences with the intended result and accuracy (sensitivity and specificity) for six biochemical tests performed

	Vibriostatic O/129 (10 μg)	8% NaCl tolerance	Citrate utilization	Ornithine decarboxylase	Arabinose fermentation	D-Mannose assimilation
Positive results	10 (21)	16 (21)	14 (21)	16 (21)	17 (21)	17 (21)
Negative results	2 (11)	2 (11)	4 (11)	5 (11)	9 (11)	7 (11)
False negative	11	5	7	5	4	4
False positive	9	9	7	6	2	4
Sensitivity %	48	76	67	76	81	81
Specificity %	18	18	36	45	82	64

citrate utilization, confirming the heterogeneity of intraspecific profiles for the *Vibrionaceae* already referred (Austin & Lee, 1992; Austin *et al*., 1997; Thompson *et al*., 2004 and references therein) and highlighted the poor accuracy of the biochemical methods. Furthermore, the urease production phenotype, considered as a virulence marker because it is reported as typical for *V. parahaemolyticus* isolates from clinical samples (Okuda *et al*., 1997), was only detected for one strain (#PVP408), while PCR assays targeting virulence genes allowed the detection of three potential pathogenic strains and underlined the unusual occurrence of *trh*-positive *V. parahaemolyticus* strains (only 0.3–3% in the total *V. parahaemolyticus* environmental population) (Caburlotto *et al*., 2008 and the reference therein), in agreement with Ottaviani *et al*. (2005).

Our results provided a different occurrence of *V. parahaemolyticus* in the two investigated sites: only six strains were collected in the C1 station during September, while the D2 station showed the highest presence of the organism (15 strains including the *trh*-positive strains), with a seasonal pattern characterized by its presence in June and during the summer–fall season (September and October). The data on *V. parahaemolyticus* distribution presented are not in agreement with those of other Italian researchers (Croci *et al*., 2001; Ottaviani *et al*., 2005), who reported a high frequency of isolation during warmer months.

In conclusion, the data presented in the present study highlight the spreading of pathogenic properties among the environmental *V. parahaemolyticus* and suggest the need for a specific monitoring plan in fisheries and bathing areas, along Northern Adriatic coasts, in order to better evaluate the real risk posed to public health.

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