Food Microbiology 84 (2019) 103258

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Clonal relationship among *Vibrio parahaemolyticus* isolated from Mediterranean mussels (*Mytilus galloprovincialis*) and grooved carpet shells (*Ruditapes decussatus*) harvested in Sardinia (Italy)

Sonia Lamon[a], Asmine Bastardo[b,1], Domenico Meloni[a,*], Simonetta Gianna Consolati[a], Federica Fois[a], Gabriella Porcheddu[a], Vanessa Agus[a], Margherita Pes[a], Maria Giovanna Cambula[a], Anna Mureddu[a], Jesus L. Romalde[b]

[a] Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Italy
[b] Departamento de Microbiologia y Parasitologia, Centro de Investigationes Biologicas (CIBUS), Faculty of Biology, Universidad de Santiago de Compostela, Spain

ARTICLE INFO

Keywords:
Shellfish
Vibrio
BOX PCR
REP PCR
ERIC PCR

ABSTRACT

The aim of the present study was to investigate the genetic variability of *Vibrio parahaemolyticus* strains isolated from naturally contaminated Mediterranean mussels (*Mytilus galloprovincialis*) and Grooved carpet shells (*Ruditapes decussatus*) from three harvesting areas of Sardinia (Italy) using a combination of different typing methods: traditional phenotypic systems and molecular techniques. Ninety-nine putative *V. parahaemolyticus* strains isolated from shellfish collected before and after purification were included in the study. Seventy-two isolates were confirmed as *V. parahaemolyticus* and were submitted to REP, ERIC and BOX PCRs. The combined dendrogram showed the similarity of the data set of the three typing methods and demonstrates how the different techniques grouped the strains in two clusters in accordance with each singular dendrogram. Several strains rendered a unique pattern regardless of the typing method, which indicates the high discriminatory power of the methods. Moreover, the use of multiple typing methods allowed a more accurate characterization of the genetic profiles of isolates and the identification of clones hardly revealed through the common techniques. The intraspecific typing of environmental *V. parahaemolyticus* can be of great interest in order to recognize clonal relationships between environmental contamination, foodborne disease, and geographical/temporal distribution of this pathogen. The comparative analysis focusing on the obtained genetic profiles supports the possibility for typing methods to discriminate strains with similar phenotypic profile, identifying the level of genetic correlation among the strains and the presence of genetic clones.

1. Introduction

Vibrio parahaemolyticus strains carrying the virulence genes associated with enteropathogenicity (*tdh* and/or *trh*) are considered pathogenic to human (Leoni et al., 2016; Nishibuchi and Kaper, 1995; Suffredini et al., 2014; Zhang and Austin, 2005; Zhang and Orth, 2013). *V. parahaemolyticus* is responsible for many food poisoning cases in some countries, *e.g.* USA and Japan (Alam et al., 2002; Carraro et al., 2015; Newton et al., 2012; Kaysner and DePaola, 2000). In Europe, only a few outbreaks or sporadic cases caused by *V. parahaemolyticus* were reported in the last decade (Martinez-Urtaza et al., 2004, 2005; 2013; McLaughlin et al., 2005; Ottaviani et al., 2008, 2009; 2010, 2012a; Quilici et al., 2005; Wootipoom et al., 2007). *V. parahaemolyticus* is usually found in estuarine, marine and coastal environments in a free-swimming state with its motility conferred by a single polar flagellum or affixed to inert and animate surfaces including zooplankton, fish, shellfish or any suspended matter underwater (Baffone et al., 2006; Gode-Potratz et al., 2011). *V. parahaemolyticus* infection is associated with the consumption of raw or under-cooked shellfish and seafood (Newton et al., 2012; Zarei et al., 2012) or exposure of skin wounds to contaminated sea water. Salinity is the main key factor influencing the occurrence of pathogenic *Vibrio* species (Caburlotto et al., 2012; Martinez-Urtaza et al., 2008). In winter months, with unfavourable water temperatures, *V. parahaemolyticus* may be undetectable (Passalacqua et al., 2016; Sferlazzo et al., 2018; Su and Liu, 2007). Pathogenic strains might have a selective advantage in surviving colder

* Corresponding author. Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Via Vienna 2, 07100, Sassari, Italy.
E-mail address: dmeloni@uniss.it (D. Meloni).
[1] Present address: Estación de Investigaciones Hidrobiológicas de Guayana, Fundación La Salle de Ciencias Naturales, 8051 Venezuela.

https://doi.org/10.1016/j.fm.2019.103258
Received 26 November 2018; Received in revised form 31 May 2019; Accepted 2 July 2019
Available online 06 July 2019
0740-0020/

conditions in marine sediments or they would be the first strains to have appeared in the environment and are gradually being replaced by non-pathogenic strains, as the water warms (DePaola et al., 2003; Rodriguez-Castro et al., 2010). A significant obstacle for the study of emergent pathogenic strains of *V. parahaemolyticus* is the lack of understanding of factors that define virulence and that could be used to detect pathogens within mostly non-pathogenic populations (Xu et al., 2015). A few of the diagnostic markers most commonly used to define pathogens are implicated in disease, including two genes associated with enteropathogenicity (*tdh* and *trh*) and a horizontally acquired type-three secretion system (T3SS2) (Banerjee et al., 2014; García et al., 2009; Hiyoshi et al., 2010; Thongjun et al., 2013). The presence of pathogenic strains in the environment does not often correlate with the incidence of food poisoning cases (Johnson et al., 2012; Jones et al., 2012). Molecular methods have given significant advances over conventional phenotypic methods to the identification and differentiation of closely related species allowing a deeper understanding of the epidemiology of *V. parahaemolyticus* strains. In literature, different genetic approaches for the identification of *Vibrio* species have been proposed (Blackstone et al., 2003; Gubala, 2006; Kim et al., 1999; Nandi et al., 2000; Panicker et al., 2004; Takahashi et al., 2005). Vibrios seem to have fewer mobile genetic elements, *e.g.,* transposons and phages, and DNA regions with a different content of G + C in comparison with the whole-genome average, which are indicative of recent horizontal transfer contributing to the plasticity of vibrio genomes (Heidelberg et al., 2000; Makino et al., 2003). The most common strain typing methods including Pulsed Field Gel Electrophoresis (PFGE), Intergenic Spacer Region (ISR-1), and Restriction Fragment Length Polymorphisms (RFLP) have been used to group similar *V. parahaemolyticus* strains (Banerjee et al., 2014; Chowdhury et al., 2004; Jones et al., 2012; Lüdeke et al., 2014; Nair et al., 2007). Multi-locus sequence analysis (MLSA) of conserved housekeeping genes can better define relatedness and recombination among strains (Banerjee et al., 2014; DePaola et al., 2003; Ellis et al., 2012; Gonzalez-Escalona et al., 2011; Jolley et al., 2004; Martinez- Urtaza et al., 2013; Paranjpye et al., 2012; Sawabe et al., 2007; Thompson et al., 2005; Turner et al., 2013). Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, Repetitive Extragenic Palindromic (REP)-PCR and Highly Conserved Repetitive DNA Elements (BOX)-PCR have been successfully applied to investigate the genetic variability among *V. parahaemolyticus* strains (Kingston et al., 2009; Maluping et al., 2005; Rao and Surendran, 2010; Rodriguez et al., 2006; Silva-Rubio et al., 2008; Staley and Harwood, 2010; Yoke-Kqueen et al., 2013; van Belkum et al., 2001). These methods revealed good discriminative ability and can be used as rapid tools to compare *Vibrio* strains in epidemiological investigations. Most of these methods have some limitations for resolving evolutionary relationships, especially when used individually: not a single method is sufficient to unambiguously examine the genetic relatedness among the endemic environmental *V. parahaemolyticus* population (Marshall et al., 1999). Sometimes, single analyses did not provide sufficient resolution or gave conflicting results. Therefore, a combination of methods may be required to achieve the complete typing of different *V. parahaemolyticus* strains, improving phylogenetic accuracy and resolution (Wong and Lin, 2001). In the present study, we have studied ninety-nine strains *V. parahaemolyticus* strains using a combination of phenotypic and molecular techniques to investigate the diversity and the relationship between *V. parahaemolyticus* isolated from naturally contaminated Mediterranean mussels (*Mytilus galloprovincialis*) and Grooved carpet shells (*Ruditapes decussatus*) harvested in Sardinia (Italy).

2. Materials and methods

2.1. Bacterial strains

Putative *V. parahaemolyticus* strains were isolated from naturally contaminated Mediterranean mussels (*M. galloprovincialis*) and Grooved carpet shells (*R. decussatus*) samples collected before and after purification from three harvesting areas (respectively, A, O and S) of Sardinia (Italy). Purification centres annexed to these production areas can be described as “recirculating”systems. In all the plants short purification protocols of 8 h were carried out. Shellfish were placed in high density polyethylene (HDPE) tanks supplied by water disinfected by ozone and/or UV. A monthly sampling was carried out from April 2011 to May 2012. Seasons were considered as: summer (July–September); autumn (October–December); winter (January–March) and spring (April–June). Recordings of environmental parameters (temperature, pH and salinity) of the water were included. Twenty-five grams of flesh and intra-valvular liquid from each sample were added to 225 ml of Alcaline Peptone Water (APW, Sigma Aldrich, Saint Louis, MO, USA), supplemented with NaCl 1%, and then homogenized in a stomacher Lab-Blender 400 (Seward Medical, London, UK) for 2 min. Detection and isolation of *Vibrio* spp was carried out plating 1 ml of each shellfish homogenate in APW (NaCl 1%) onto the surface of CHROMagar (Oxoid) and Modified Cellobiose-Polymyxin B-Colistin (mCPC, Oxoid) Agar plates. All the plates were incubated at 37 °C for 48 h. Presumptive identification of *Vibrio* spp. isolates was performed by means of conventional biochemical tests: five colonies randomly selected from the plates of each medium were streaked on 3% NaCl tryptone soya agar plates (TSA-s, Oxoid) and incubated at 30 °C for 24 h. The colonies on TSA-s were selected for typical appearance (convex, light yellow and opaque, with a diameter between 1 and 2 mm) and successively screened through oxidase and catalase tests, Gram staining, sugar fermentation and sensitivity to vibriostatic agent O129. Biochemical identification was carried out by the API20NE identification system (bioMérieux, Marcy l’Etoile, France).

2.2. Molecular identification and virulence profile of V. parahaemolyticus

Firstly, *V. parahaemolyticus* isolates identified by phenotypic and biochemical methods have been submitted to molecular identification by means of a single polymerase chain reaction (s-PCR) assay targeting the *tox*R gene as described by Bej et al. (1999). Virulotyping of *V. parahaemolyticus* isolates was performed by means of two different s-PCR assays (s-PCR 1 and s-PCR 2) targeting the virulence genes associated with enteropathogenicity (*tdh* and *trh*) according to the protocols of Bej et al. (1999). S-PCR 1 aimed to amplify the *trh* gene to identify thermostable direct haemolysin-related and s-PCR 2 aimed to amplify the *tdh* gene to identify thermostable direct haemolysin. Positive (*V. parahaemolyticus* ATCC 43996 *tox*R +, *tdh* +, *trh*; *V. parahaemolyticus*ATCC 17802 toxR +, tdh -, trh +) and negative controls (*Listeria* spp. strain from internal collection of University of Sassari) were included in all s-PCR tests. All PCR amplifications were performed by using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, US). PCR products were separated by electrophoresis on 1.2% (w / v) agarose gel and visualized under ultraviolet light after ethidium bromide (0.1 mg/ml) staining. The gel images were visualized by Quantity-One software (Bio-Rad, Hercules, CA, USA) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad). All the isolates confirmed as *V. parahaemolyticus* by biomolecular tests were submitted to genotyping characterization.

2.3. Genotypic characterization

V. parahaemolyticus isolates confirmed by PCR tests were submitted to Repetitive Extragenic Palindromic (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC) and highly conserved repetitive DNA element (BOX) PCRs. Genomic DNA was extracted from pure bacterial cultures using Insta-Gene matrix (Bio-Rad, Hercules, CA, USA). The concentration of DNA was quantified spectrophotometrically and adjusted to a concentration of 100 ng/μl. For the ERIC, REP and BOX-PCR analysis, a reference strain (*V. parahaemolyticus* ATCC 43996 (*tox*R +, *tdh* +, *trh* -) was included in each PCR test. REP PCR was performed in

Table 1
Vibrio parahaemolyticus strains ($n = 24$) isolated from Mediterranean mussels in relation with season, harvesting area and environmental parameters of seawater.

Date	Harvesting area	Water temperature (°C)	Water salinity (ppt)	Water pH	Strain code	*toxR*	*tdh*+	*trh*+
May 2011	A	19.64	40	8.72	B1	+	–	–
June 2011	S	21.29	33	8.37	C2	+	–	–
July 2011	A	22.81	40	7.93	D1	+	–	–
July 2011	A	22.81	40	7.93	D12[a]	+	–	–
July 2011	A	22.81	40	7.93	D18[a]	+	–	–
Sept. 2011	A	23.7	40	7.92	E10	+	–	–
Sept. 2011	S	22.94	36	8.55	E11	+	–	–
Sept. 2011	S	22.94	36	8.55	E12	+	–	–
Nov. 2011	A	17.86	39	7.58	G93	+	–	–
Nov. 2011	A	17.86	39	7.58	G94	+	–	–
Nov. 2011	A	17.86	39	7.58	G109[a]	+	–	–
Nov. 2011	A	17.86	39	7.58	G110[a]	+	–	–
Nov. 2011	A	17.86	39	7.58	G111[a]	+	–	–
Nov. 2011	A	17.86	39	7.58	G115[a]	+	–	–
Dec. 2011	A	13.12	38	9.53	H39	+	–	–
Dec. 2011	A	13.12	38	9.53	H42	+	–	–
Dec. 2011	A	13.12	38	9.53	H120	+	–	–
Dec. 2011	A	13.12	38	9.53	H25	+	–	–
Dec. 2011	A	13.12	38	9.53	H45	+	–	–
Dec. 2011	A	13.12	38	9.53	H40	+	–	–
Dec. 2011	A	13.12	38	9.53	H32	+	–	–
Dec. 2011	A	13.12	38	9.53	H30	+	–	–
Dec. 2011	A	13.12	38	9.53	H48	+	–	–
Dec. 2011	A	13.12	38	9.53	H10	+	–	–

[a] Isolated from samples collected after purification.

accordance with Versalovic et al. (1991). The two primers that anneal the so-called repetitive DNA elements distributed more or less randomly over the genome were: REP1D (5′-3′NNNRCGYCGNCATCMGGC) and REP2D (5′-3′AGCGCTCTTATCACGGCCTAC). ERIC-PCR fingerprinting was carried out with two repetitive primer sequences ERIC1R (5′-3′ ATGTAAGCTCCTGGGGATTCA) and ERIC2 (5′–3′AAGTAAGTGACTGG GGTGAGCG) according to Versalovic et al. (1991). BOX-PCR was carried out using highly conserved repeated DNA elements according to Versalovic et al. (1994). The single oligonucleotide employed was BOX A1R *(5′-CTACGGCAAGGCGACGCTGACG-3′)*. PCR amplifications were performed by using a Mastercycler Engine DNA Thermal Cycler (Eppendorf). PCR products were separated by electrophoresis on 1% (w / v) agarose gel and visualized under ultraviolet light after ethidium bromide (0.5 μg/ml) staining. The gel images were visualized by Quantity-One software (Bio-Rad) and captured using the Gel-Doc UV trans-illuminator (Bio-Rad). Gel images were saved as TIFF files, normalized with the FastRuler™ Ladder 1 kb molecular weight marker. Molecular patterns were analyzed using the Diversity Database software (Bio-Rad).

2.4. Polyphasic analysis

To obtain the diversity index for each typing method and their different combinations, clustering and construction of different dendrograms were performed through the Unweighted Pair Group Method using arithmetic averages (UPGMA) and a similarity value of 70%. Data of harvest, origin of samples and genotyping (ERIC-REP-BOX PCR patterns) were introduced and analyzed using the software package Bionumerics v. 6.6.4 (Applied Maths NV, Sint-Martens-Latem, BE).

2.5. PCR of subunit 16s rDNA

A selection of isolates not confirmed as *V. parahaemolyticus* was sequenced using 16s ribosomal RNA. According to Lane (1991), the following PCR primers were used to amplify the 16S rRNA gene providing the phylogenetic information: 27FY *(5′- AGAGTTTGATCMTGG CTCAG-3′)* and 1510R *(5′-TACGGYTACCTTGTTACGACTT-3′)*. Ready-To-Go Pcr Beads (GE Healthcare Europe GmbH, Freiburg, GE) were used to set a conventional PCR to amplify 16s rRNA gene. Each Ready-To-Go Pcr Beads tube contained 1 μl of the suspension of extracted template DNA, sterile distilled water and 1 μl of each primer. PCR amplifications were performed by using an Eppendorf Mastercycler Engine DNA Thermal Cycler (Eppendorf, Hamburg. Ge). PCR products were separated by electrophoresis on 1.5% (w / v) agarose gel and were dispatched to STAB VIDA laboratories (Universidade Nova de Lisboa-Monte da Caparica Campus-Life Sciences Department). The sequences were analyzed using the software DNASTAR Lasergene. The application SeqMan was used for counting, assembly and analysis while EditSeq for importing and editing file types. BLAST (http://blast.ncbi.nlm.nih.gov/) and EzTaxon (http://147.47.212.35:8080/index.jsp) were used to compare nucleotide sequences with sequences databases and calculate the statistical significance of matches.

3. Results

3.1. Phenotypic and biochemical identification of V. parahaemolyticus strains

A total of ninety-nine putative *V. parahaemolyticus* strains have been submitted to presumptive phenotypic and biochemical identification. Seventy strains were isolated from *R. decussatus* and twenty-nine strains from *M. galloprovincialis*. Altogether, eighty-six isolates have been identified as *V. parahaemolyticus* by phenotypic and biochemical methods and were submitted to biomolecular confirmation and virulotyping.

3.2. Molecular identification and virulence profile of V. parahaemolyticus

The eighty-six isolates confirmed as *V. parahaemolyticus* by biochemical methods were submitted to genotyping characterization. Altogether, seventy-two isolates were confirmed as *V. parahaemolyticus.* Twenty-four strains were isolated from Mediterranean mussels and forty-eight strains from Grooved carpet shells (Tables 1 and 2). Among these, sixty-five strains were isolated before and seven after purification. Potentially pathogenic *V. parahaemolyticus* strains were a minority: two strains carried the *tdh* gene associated to thermostable direct haemolysin-and one strain carried the *trh* gene associated to thermostable direct haemolysin-related. All these strains have been isolated

Table 2
Vibrio parahaemolyticus strains ($n = 48$) isolated from Grooved carpet shells in relation with season, harvesting area and environmental parameters of seawater.

Date	Harvesting Area	Water temperature (°C)	Water salinity (ppt)	Water pH	Strain code	*toxR*	*tdh*+	*trh*+
May 2011	A	19.6	40	8.7	B14	+	+	–
June 2011	A	21.5	40.12	8.2	C23	+	–	–
June 2011	O	22.5	33.1	8.3	C4	+	–	–
July 2011	A	22.8	40.1	7.9	D25	+	–	–
July 2011	A	22.8	40.1	7.9	D26	+	–	–
July 2011	A	22.8	40.1	7.9	D28	+	–	–
July 2011	O	24.5	34.9	8.3	D56	+	–	–
July 2011	O	24.5	34.9	8.3	D8	+	–	–
Sept. 2011	A	23.7	40.5	7.9	E13	+	–	–
Sept. 2011	A	23.7	40.5	7.9	E15	+	–	–
Sept. 2011	A	23.7	40.5	7.9	E16	+	–	–
Sept. 2011	A	23.7	40.5	7.9	E17	+	–	–
Sept. 2011	A	23.7	40.5	7.9	E28	+	–	–
Sept. 2011	A	23.7	40.5	7.9	E54	+	–	–
Oct. 2011	A	20.8	40.1	7.9	F12	+	–	–
Oct. 2011	A	20.8	40.1	7.9	F13	+	–	–
Oct. 2011	A	20.8	40.1	7.9	F14	+	–	–
Oct. 2011	A	20.8	40.1	7.9	F15	+	–	–
Oct. 2011	A	20.8	40.1	7.9	F16	+	–	–
Oct. 2011	A	20.8	40.1	7.9	F32	+	–	–
Oct. 2011	O	20.5	38	8.6	F33	+	–	–
Oct. 2011	O	20.5	38	8.6	F66	+	–	–
Oct. 2011	O	20.5	38	8.6	F67	+	–	–
Oct. 2011	O	20.5	38	8.6	F74	+	+	–
Oct. 2011	O	20.5	38	8.6	F75	+	–	–
Nov. 2011	A	17.9	39.5	7.6	G12	+	–	–
Nov. 2011	A	17.9	39.5	7.6	G13	+	–	–
Nov. 2011	A	17.9	39.5	7.6	G14	+	–	–
Nov. 2011	A	17.9	39.5	7.6	G26[a]	+	–	–
Nov. 2011	O	16	27	8.3	G41	+	–	–
Dec. 2011	A	13.1	38.9	9.5	H17	+	–	–
Dec. 2011	A	13.1	38.9	9.5	H21	+	–	–
Dec.2011	A	13.1	38.9	9.5	H41	+	–	–
Dec. 2011	A	13.1	38.9	9.5	H37	+	–	–
Dec. 2011	A	13.1	38.9	9.5	H44	+	–	–
Dec. 2011	A	13.1	38.9	9.5	H16	+	–	–
Dec. 2011	A	13.1	38.9	9.5	H12	+	–	–
Dec. 2011	A	13.1	38.9	9.5	H20	+	–	–
Dec. 2011	A	13.1	38.9	9.5	H3	+	–	+
Dec. 2011	A	13.1	38.9	9.5	H9	+	–	–
Dec. 2011	O	16	36.4	8.3	H54	+	–	–
Dec. 2011	O	16	36.4	8.3	H55	+	–	–
Dec. 2011	O	16	36.4	8.3	H56	+	–	–
Dec. 2011	O	16	36.4	8.3	H57	+	–	–
Dec. 2011	O	16	36.4	8.3	H58	+	–	–
Dec. 2011	O	16	36.4	8.3	H59	+	–	–
Dec. 2011	O	16	36.4	8.3	H62	+	–	–
Dec. 2011	O	16	36.4	8.3	H63	+	–	–

[a] Isolated from samples collected after purification.

from Grooved carpet shells sampled before purification. Not a single strain was *tdh*+/*trh*+.

3.3. Genotypic characterization

The seventy-two isolates confirmed as *V. parahaemolyticus* by conventional PCR were further characterized by ERIC-PCR, REP-PCR and BOX-PCR methods. Genotyping by ERIC-PCR allowed the identification of profiles having 9–13 amplification bands, ranging from 90 to 2780 kb (Fig. 1). Genotyping by REP-PCR allowed the identification of profiles having 9–15 amplification bands, ranging from 102 to 2128 kb (Fig. 2) while genotyping by BOX-PCR allowed the identification of profiles having 8–10 amplification bands, ranging from 98 to 2780 kb (Fig. 3).

3.4. Polyphasic analysis

The clonal relationship among the seventy-two *V. parahaemolyticus* strains were examined through UPGMA-cluster analysis of the PCR-generated profiles and are presented in dendrograms. The ERIC-PCR profiles were firstly allotted into four groups (similarity ≥ 70%) labelled as I-II-III-IV (Fig. 1). Dendrogram patterns produced two major clusters designated as A and B (Fig. 1). Cluster A consisted of 23 strains from areas A and O, while Cluster B represented 49 isolates from areas A, O and S. The dendrogram pattern generated from REP-PCR (Fig. 2) produced two major clusters designated as A and B, consisting of 15 and 57 strains respectively. Cluster A grouped strains from rearing sites A and O. Cluster B strains, from sites A, O and S, were allotted into two groups (similarity ≥ 70%) labelled as I-II. The dendrogram pattern generated from BOX-PCR (Fig. 3) produced two major clusters designated as A (7 strains) and B (65 strains). Cluster A and B grouped isolates from rearing A, O and S. The combined data set of the three typing methods (Fig. 4) grouped the strains in 2 clusters (A and B) in accordance with each singular dendrogram. Cluster A consisted of 42 strains isolated in autumn, while the distribution of the remaining 30 isolates in cluster B was more heterogeneous. Interestingly, strains isolated in summer grouped together.

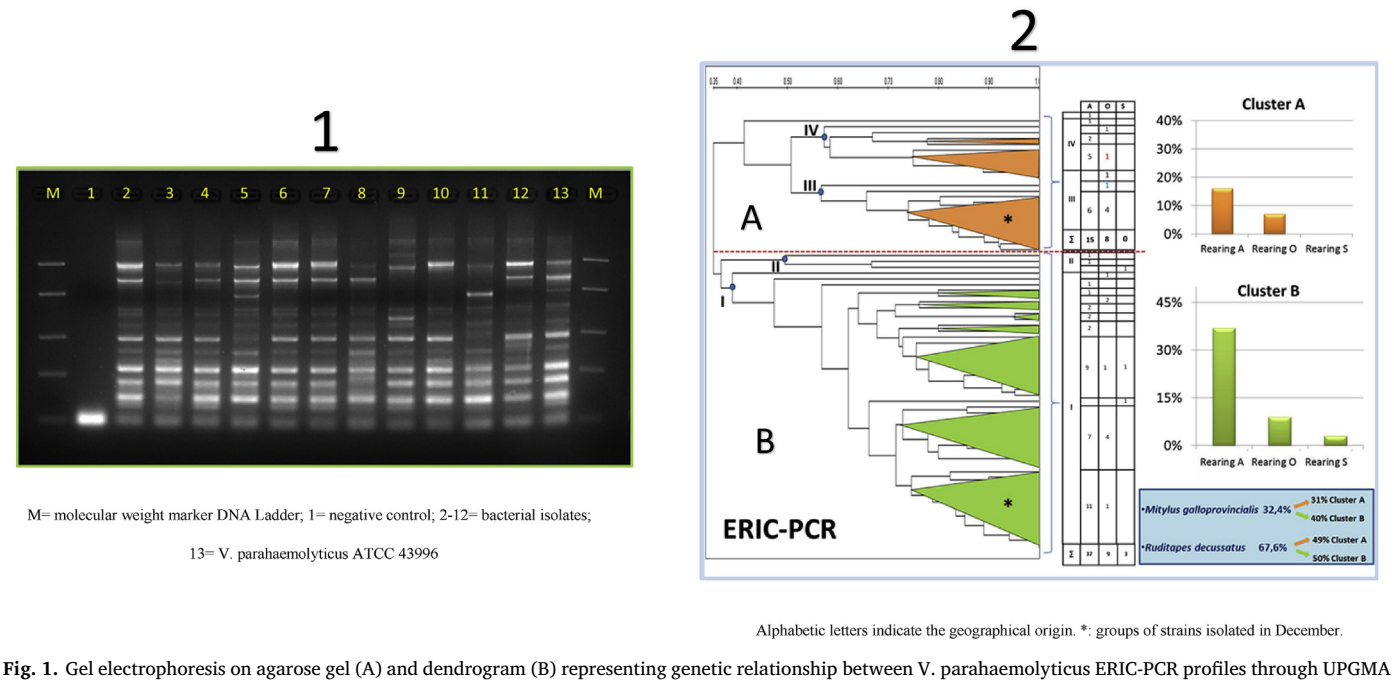

Fig. 1. Gel electrophoresis on agarose gel (A) and dendrogram (B) representing genetic relationship between V. parahaemolyticus ERIC-PCR profiles through UPGMA clustering.

3.5. PCR of subunit 16s rDNA

The fourteen isolates not confirmed as *V. parahaemolyticus*, were compared to select the most similar according to the profile bands in the s-PCR. Four strains were selected (F1, D2, F73 and H13)and sequenced using 16s ribosomal RNA to clarify their phylogenetic structure. Several *Vibrionaceae* species have nearly identical 16S rRNA gene sequences (Table 3). Two different databases (BLAST and EzTaxon) showed high similarity values between *V. parahaemolyticus* (99%) and other different species (*V. alginolyticus* 99%, *V. azureus* 99%, *V. owensii* 99%, *V. natriegens* 99%). Seven species appeared to be closely related, sharing an overall level of sequence similarity of 91.3% (*V. alginolyticus, V. campbellii, V. harveyi, V. natriegens, V. parahaemolyticus, V. vulnifcus and V. proteolyticus*).

4. Discussion

This study provided a different occurrence of *V. parahaemolyticus* in the different investigated hosts, sites and seasons: eighty-six isolates identified as *V. parahaemolyticus* by phenotypic and biochemical methods were submitted to biomolecular confirmation and virulotyping. Seventy-two isolates (84%) were confirmed as *V. parahaemolyticus* mostly isolated from *R. decussatus:* they are usually harvested in muddy-sand sediments of shallow coastal waters, while *M.*

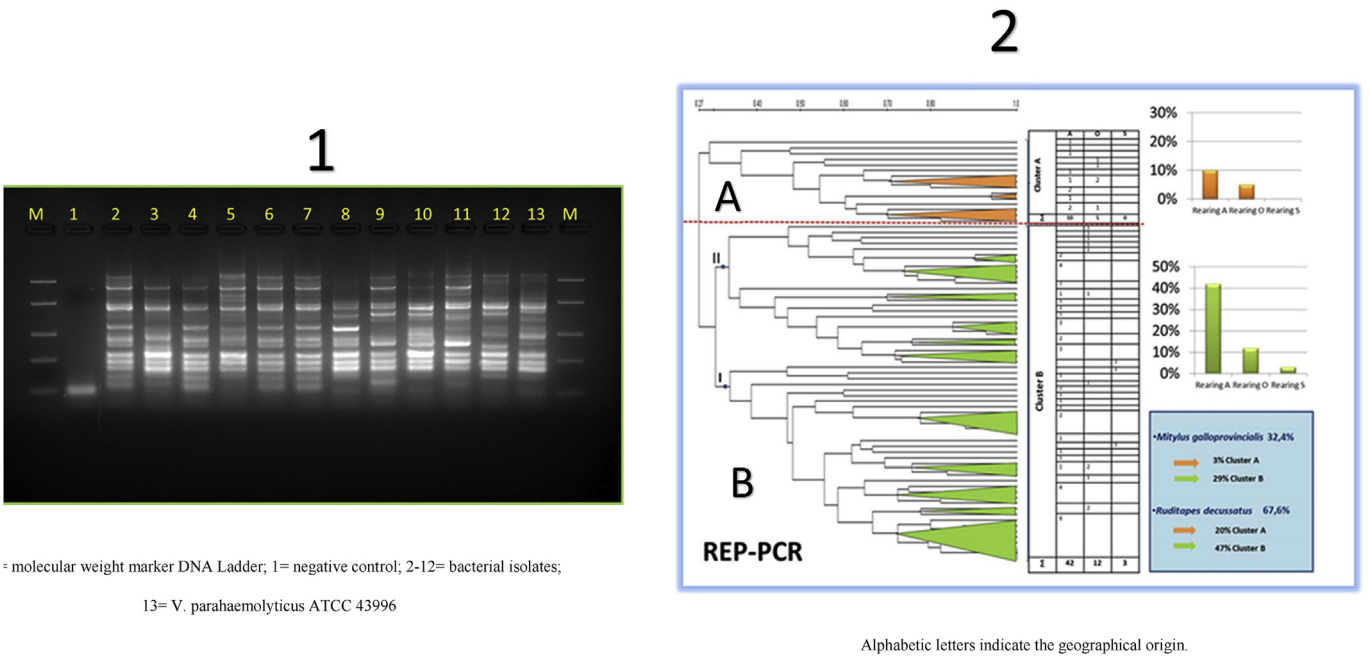

Fig. 2. Gel electrophoresis on agarose gel (A) and dendrogram (B) representing genetic relationship between *V. parahaemolyticus* REP-PCR profiles through UPGMA clustering.

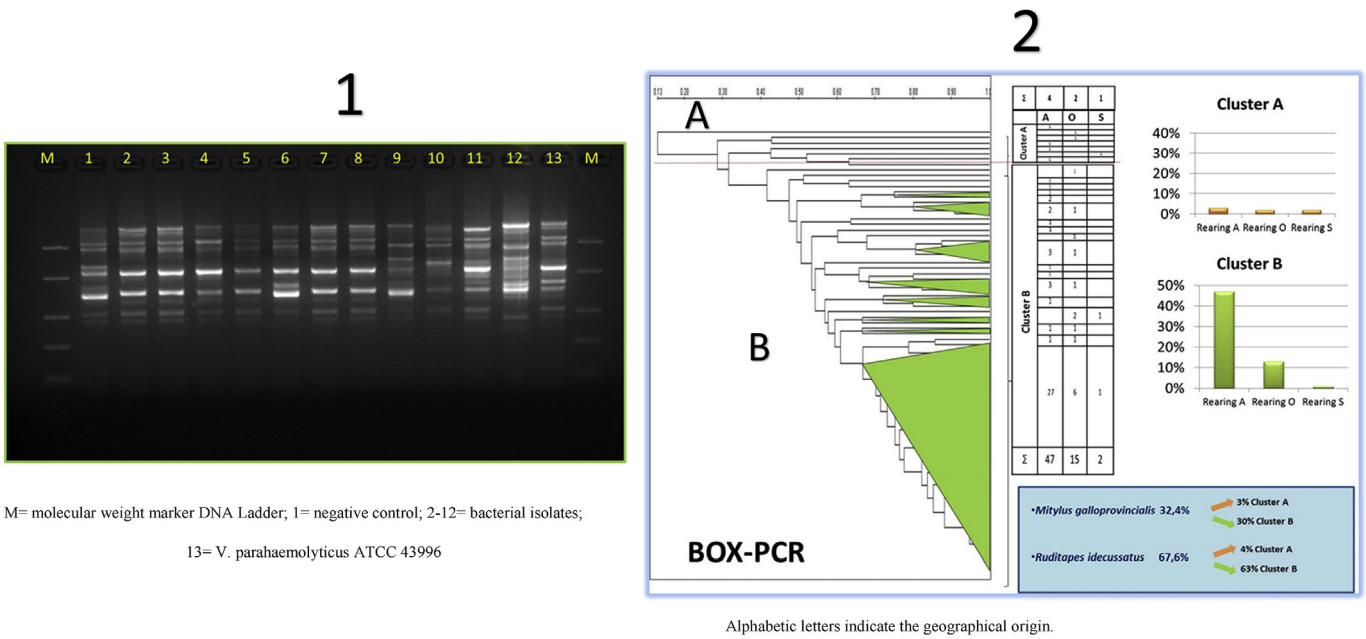

Fig. 3. Gel electrophoresis on agarose gel (A) and dendrogram (B) representing genetic relationship between *V. parahaemolyticus* BOX-PCR profiles through UPGMA clustering.

galloprovincialis are harvested on suspended ropes. These results were in contrast with previous studies about the distribution of *V. parahaemolyticus* in Italian marine coastal waters (Ottaviani et al., 2012b).

Potentially toxigenic *V. parahaemolyticus* strains (*i.e.* carrying *tdh* and/or *trh* genes) were recovered in low numbers: the prevalence of each virulent factor was 0.9%. According to Carraro et al. (2015), in

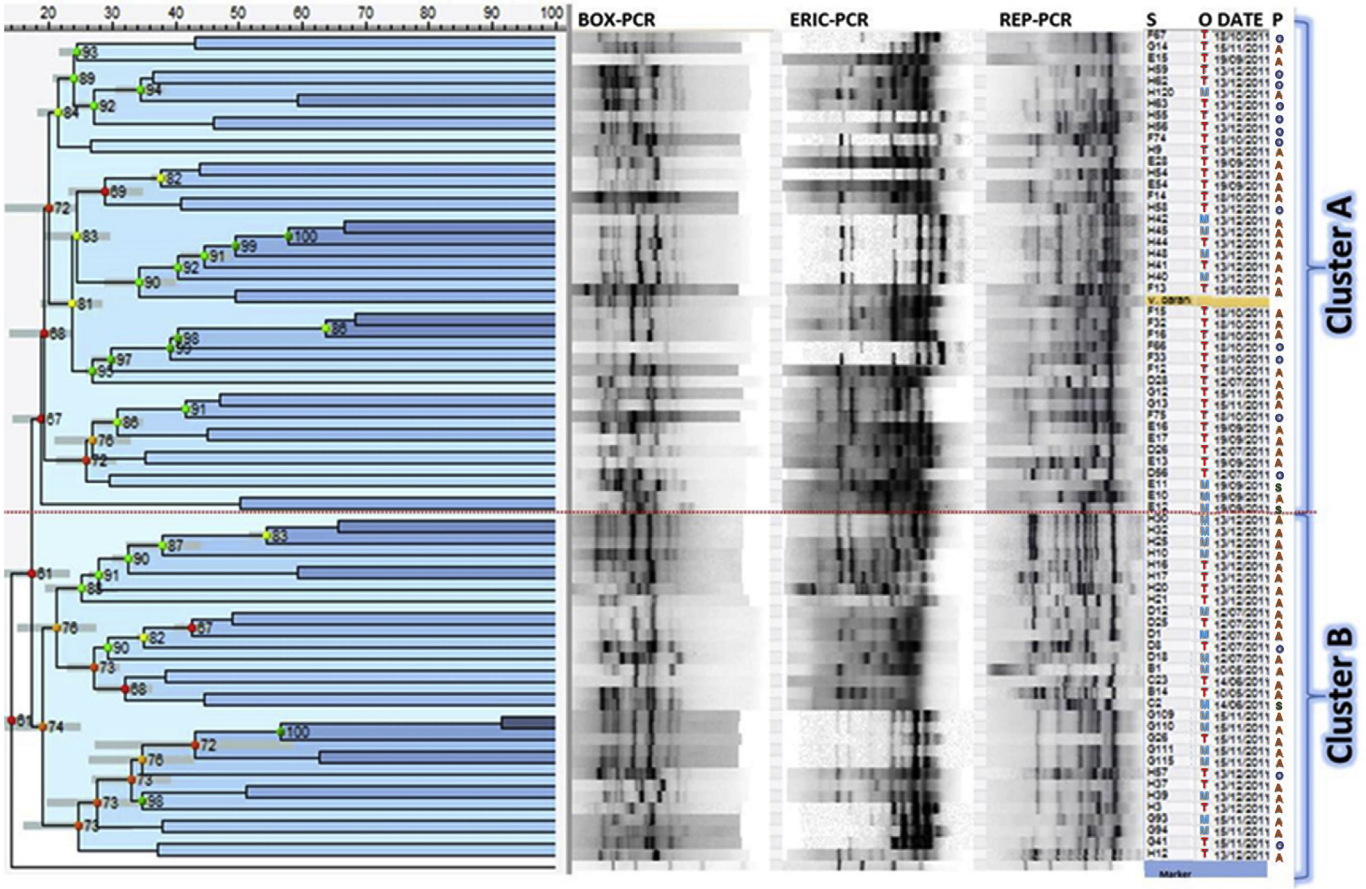

Fig. 4. Combined dendrogram representing phylogenetic relationship between V. parahaemolyticus ERIC-PCR, REP-PCR and BOX-PCR profiles through UPGMA clustering.

Sardinian production areas the presence of potential pathogenic *V. parahaemolyticus trh* + strains is very low, and it is unrelated to the total vibrio population (Johnson et al., 2012). These results suggest that potentially pathogenic *V. parahaemolyticus* strains transited in Sardinian coastal habitats only during a limited period and are found extremely rarely or they are unlikely detectable. In literature, the prevalence of pathogenic *V. parahaemolyticus* ranges from 1% to 33% (Bauer et al., 2006; DePaola et al., 2003; Deter et al., 2010; Di Pinto et al., 2008; Hervio-Heath et al., 2002; Nishibuchi and Kaper., 1995; Ottaviani et al., 2010; Passalacqua et al., 2016; Robert-Pillot et al., 2004; Rodriguez-Castro et al., 2010; Wagley et al., 2008; Serracca et al., 2011; Suffredini et al., 2014). The seventy-two isolates confirmed as *V. parahaemolyticus* by conventional PCR were subjected to PCR-based fingerprinting with short random primers or primers directed against repetitive sequences in the bacterial genome (Colombo et al., 1997 Maluping et al., 2005; Olive and Bean, 1999; Rodriguez et al., 2006; Versalovic et al., 1991). REP-PCR and ERIC-PCR methods revealed a good discriminative ability (Wong and Lin, 2001) in comparison with BOX-PCR and showed the high heterogeneity existing among the *V. parahaemolyticus strains.* This was proved by the highest number of different DNA fragment patterns (33 patterns with REP-PCR, 31 patterns with ERIC-PCR, and 30 with BOX-PCR). The combined dendrogram showed the similarity of the data set of the three typing methods and demonstrates how the different techniques grouped the strains in two clusters (A and B) in accordance with each singular dendrogram. Cluster A grouped strains isolated mainly in autumn, while the distribution of the isolates in cluster B was more heterogeneous. In our study, the greater number of *V. parahaemolyticus* strains were isolated on December 2011 in comparison with the isolates of the other months. In general, the data on *V. parahaemolyticus* distribution were not in agreement with those of other Italian researchers (Croci et al., 2001; Ottaviani et al., 2010) who reported a high frequency of isolation during warmer months. According to ERIC and REP PCR, the majority of strains allotted in the cluster A, including *trh* + and *tdh* + isolates, showed a clear temporal distribution, grouping all the strains isolated in autumn. The strains allotted in cluster B were more irregular in terms of sampling period and host. According to ERIC PCR, the *V. parahaemolyticus* strains isolated on December 2011 clustered in B as in A. BOX-PCR did not show a close relationship between the clusters and the sampling seasons. Several genetic profiles persisted over time: according to Caburlotto et al. (2012) strains showing very similar genetic patterns were isolated for two or three subsequent months. The strains B14 and F74 (*tdh*+) were allotted in the same cluster (A) with ERIC-PC. Virulence genes can be acquired, via horizontal genetic transfer (Caburlotto et al., 2012): BOX-PCR method grouped the strains B14, F74 (*tdh*+) and H3 (*trh*+) in the cluster B. REP-PCR instead, allotted the strains B14 (*tdh*+) and H3 (*trh* +) in the cluster B. Several profiles suggested a close relationship between isolates and harvesting area. The three samples carrying the genetic virulence markers *trh* and *tdh* (H3 *trh*+, B14 tdh+ and F74 tdh +) were from the same host (*R. semidecussatus*) and two of them (B14 and H3) from the same geographic area (A).

5. Conclusions

The intraspecific typing of environmental *V. parahaemolyticus* can be of great interest for the recognition of clonal relationships between environmental contamination, foodborne disease, and geographical and temporal distribution of this pathogen. Moreover, the use of multiple typing methods would allow a more accurate characterization of the genetic profiles of isolates and the identification of clones hardly revealed through the common techniques. The comparative analysis focusing on the obtained genetic profiles and the presence of virulence genes further supports the possibility for typing methods to discriminate strains with similar phenotypic profiles, identifying the level of genetic correlation among the strains and the presence of genetic clones. These typing methods have also been shown to be capable of discriminate among very similar strains isolated in the same sampling session, but in a different sampling site.

Funding source

This work was funded by: Regione Autonoma della Sardegna, L.R. 7 Agosto 2007, N. 7 "*Promozione della Ricerca Scientifica e dell'Innovazione Tecnologica in Sardegna*". Progetto di Ricerca fondamentale o di base: "*Sviluppo di tecnologie biomolecolari innovative per la sorveglianza epidemiologica di contaminanti batterici e virali ai fini della valorizzazione della produzione dei molluschi bivalvi della Sardegna*". project ID: CRP2_470.

Table 3
PCR of subunit 16s rDNA of four isolates not confirmed as *V. parahaemolyticus* with conventional PCR

Strain	Blast %	EzTaxon %
F1	*V. alginolyticus* (99%)	*V. owensii (99.44%)*
	V. azureus (99%)	
	V. parahaemolyticus (99%)	*V. azureus (99.31%)*
D2	*V. owensii (99%)*	*V. azureus (99.46%)*
	V. azureus (99%)	
	V. natriegens (99%)	*V. owensii (99.44%)*
F73	*V. azureus (99%)*	*V. owensii (99.44%)*
	V. parahaemolyticus (99%)	
	V. owensii (99%)	*V. azureus (99.31%)*
H13	*V. alginolyticus (99%)*	*V. alginolyticus (99%)*
	V. azureus (99%)	
	V. parahaemolyticus (99%)	*V. azureus (99.31%)*

References

Alam, M.J., Tomochika, K.I., Miyoshi, S.I., Shinoda, S., 2002. Environmental investigation of potentially pathogenic *Vibrio parahaemolyticus* in the Seto-Inland sea, Japan. FEMS Microbiol. Lett. 208, 83–87.

Baffone, W., Tarsi, R., Pane, L., Campana, R., Repetto, B., Mariottini, G.L., Pruzzo, C., 2006. Detection of free-living and plankton-bound vibrios in coastal waters of the Adriatic Sea (Italy) and study of their pathogenicity associated properties. Environ. Microbiol. 8, 1299–1305.

Banerjee, S.K., Kearney, A.K., Nadon, C.A., Peterson, C.L., Tyler, K., Bakouche, L., Clark, C.G., Hoang, L., Gilmour, M.W., Farber, J.M., 2014. Phenotypic and genotypic characterization of Canadian clinical isolates of *Vibrio parahaemolyticus* collected from 2000 to 2009. J. Clin. Microbiol. 52, 1081–1088.

Bauer, A., Ostensvik, O., Florvag, M., Ormen, O., Rorvik, L.M., 2006. Occurrence of *Vibrio parahaemolyticus, V. cholerae* and *V. vulnificus* in Norwegian blue mussels (*Mytilus edulis*). Appl. Environ. Microbiol. 72, 3058–3061.

Bej, A.K., Patterson, D.P., Brasher, C.W., Vickery, M.C., Jones, D.D., Kaysner, C.A., 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tlh. tdh and trh. J. Microbiol. Methods 36, 215–225.

Blackstone, G.M., Nordstrom, J.L., Vickery, M.C.L., Bowen, M.D., Mayer, R.F., DePaola, A., 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. J Microbial Methods 53, 149–155.

Caburlotto, G., Bianchi, F., Gennari, M., Ghidini, V., Socal, G., Aubry, F.B., Bastianini, M., Tafi, M., Lleo, M.M., 2012. Integrated evaluation of environmental parameters influencing Vibrio occurrence in the coastal Northern Adriatic Sea (Italy) facing the Venetian lagoon. Microb. Ecol. 63, 20–31.

Carraro, V., Sanna, C., Brandas, V., Sanna, A., Pinna, A., Coroneo, V., 2015. Hygiene and health risks associated with the consumption of edible lamellibranch molluscs. Int. J. Food Microbiol. 201, 52–57.

Chowdhury, N.R., Stine, O.C., Morris, J.G., Nair, G.B., 2004. Assessment of evolution of pandemic *Vibrio parahaemolyticus* by multilocus sequence typing. J. Clin. Microbiol. 42, 1280–1282.

Colombo, M.M., Mastrandrea, S., Leite, F., Santona, A., Uzzau, S., Rappelli, P., Pisano, M., Rubino, S., Cappuccinelli, P., 1997. Tracking of clinical and environmental *Vibrio cholerae* O1 strains by combined analysis of the presence of toxin cassette, plasmid content and ERIC PCR. FEMS Immunol. Med. Microbiol. 19, 33–45.

Croci, L., Serratore, P., Cozzi, L., Stracchini, A., Milandri, S., Suffredini, E., Toti, L., 2001. Detection of Vibrionaceae in mussels and in their seawater growing area. Lett. Appl. Microbiol. 32, 57–61.

DePaola, A., Ulaszek, J., Kaysner, C.A., Tenge, B.J., Nordstrom, J.L., Wells, J., Puhr, N., Gendel, S.M., 2003. Molecular, serological and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food and clinical sources in North America and Asia. Appl. Environ. Microbiol. 69, 3999–4005.

Deter, J., Lozach, S., Veron, A., Chollet, J., Derrien, A., Hervio-Heath, D., 2010. Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a. Environ. Microbiol. 12, 929–937.

Di Pinto, A., Ciccarese, G., De Corato, R., Novello, L., Terio, V., 2008. Detection of

pathogenic *Vibrio parahaemolyticus* in southern Italian shellfish. Food Control 19, 1037–1041.
Ellis, C.N., Schuster, B.M., Striplin, M.J., Jones, S.H., Whistler, C.A., Cooper, V.S., 2012. Influence of seasonality on the genetic diversity of *Vibrio parahaemolyticus* in New Hampshire shellfish waters as determined by multilocus sequence analysis. Appl. Environ. Microbiol. 78, 3778–3782.
García, K., Torres, R., Uribe, P., Hernández, C., Rioseco, M.L., Romero, J., Espejo, R.T., 2009. Dynamics of clinical and environmental *Vibrio parahaemolyticus* strains during seafood-related summer diarrhea outbreaks in southern Chile. Appl. Environ. Microbiol. 75, 7482–7487.
Gode-Potratz, C.J., Kustusch, R.J., Breheny, P.J., Weiss, D.S., McCarter, L.L., 2011. Surface sensing in *Vibrio parahaemolyticus* triggers a programme of gene expression that promotes colonization and virulence. Mol. Microbiol. 79, 240–263.
Gonzalez-Escalona, N., Strain, E.A., De Jesus, A.J., Jones, J.L., DePaola, A., 2011. Genome sequence of a clinical O4:K12 serotype *Vibrio parahaemolyticus* strain 10329. J. Bacteriol. 193, 3405–3406.
Gubala, A.J., 2006. Multiplex real-time PCR detection of *Vibrio cholerae*. J. Microbial. Methods. 65, 278–293.
Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D., Umayam, L., Gill, S.R., Nelson, K.E., Read, T.D., Tettelin, H., Richardson, D., Ermolaeva, M.D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R.D., Nierman, W.C., White, O., 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 406, 477–483.
Hervio-Heath, D., Colwell, R.R., Derrien, A., Robert-Pillot, A., Fournier, J.M., Pommepuy, M., 2002. Occurrence of pathogenic vibrios in coastal areas of France. J. Appl. Microbiol. 92, 1123–1135.
Hiyoshi, H., Kodama, T., Iida, T., Honda, T., 2010. Contribution of *Vibrio parahaemolyticus* virulence factors to cytotoxicity, enterotoxicity and lethality in mice. Infect. Immun. 78, 1772–1780.
Johnson, C.N., Bowers, J.C., Griffitt, K.J., Molina, V., Clostio, R.W., Pei, S., Laws, E., Paranjpye, R.N., Strom, M.S., Chen, A., Hasan, N.A., Huq, A., Noriea, N.F., Grimes, D.J., Colwell, R.R., 2012. Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi and Washington (United States). Appl. Environ. Microbiol. 78, 7249–7257.
Jolley, K.A., Chan, M.S., Maiden, M.C., 2004. mlstdbNet–distributed multi locus sequence typing (MLST) databases. BMC Bioinf. 5, 86 2004.
Jones, J.L., Ludeke, C.H.M., Bowers, J.C., Garrett, N., Fischer, M., Parsons, M.B., Bopp, C.A., DePaola, A., 2012. Biochemical, serological and virulence characterization of clinical and oyster *Vibrio parahaemolyticus* isolates. J. Clin. Microbiol. 50, 2343–2352.
Kaysner, C.A., DePaola, A., 2000. Outbreaks of *Vibrio* spp gastroenteritis from raw oyster consumption: assessing the risk of consumption and genetic methods for detection of pathogen strains. J. Shellfish Res. 19, 657–660.
Kim, Y.B., Okuda, J., Matsumoto, C., Takahashi, N., Hashimoto, S., Nishibuchi, M., 1999. Identification of *Vibrio parahaemolyticus* strain at the species level by PCR targeted to the toxR gene. J. Clin. Microbiol. 37, 1173–1177.
Kingston, J.J., Zachariah, K., Tuteja, U., Kumar, S., Batra, H.V., 2009. Molecular characterization of *Vibrio cholerae* isolates from cholera outbreaks in North India. J. Microbiol. 47, 110–115.
Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Technique in Bacterial Systematics. John Wiley & Sons, Chichester, UK, pp. 115–175.
Leoni, F., Talevi, G., Masini, L., Ottaviani, D., Rocchegiani, E., 2016. Trh (tdh − /trh +) gene analysis of clinical, environmental and food isolates of *Vibrio parahaemolyticus* as a tool for investigating pathogenicity. Int. J. Food Microbiol. 225, 43–53.
Lüdeke, C.H., Fischer, M., Lafon, P., Cooper, K., Jones, J.L., 2014. Suitability of the molecular subtyping methods intergenic spacer region, direct genome restriction analysis, and Pulsed-Field Gel Electrophoresis for clinical and environmental *Vibrio parahaemolyticus* Isolates. Foodb. Pathog. Dis. 11, 520–528.
Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M., Yamashita, A., Kubota, Y., Kimura, S., Yasunaga, T., Honda, T., Shinagawa, H., Hattori, M., Iida, T., 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. Lancet 361, 743–749.
Maluping, R.P., Ravelo, C., Lavilla-Pitogo, C.R., Krovacek, K., Romalde, J.L., 2005. Molecular typing of *Vibrio parahaemolyticus* strains isolated from the Philippines by PCR-based methods. J. Appl. Microbiol. 99, 383–391.
Marshall, S., Clark, G., Wang, G., Mulvey, M., Kelly, M.T., Johnson, W.M., 1999. Comparison of molecular methods for typing *Vibrio parahaemolyticus*. J. Clin. Microbiol. 37 (8), 2473–2478.
Martinez-Urtaza, J., Lozano-Leon, A., DePaola, A., Ishibashi, M., Shimada, K., Nishibuchi, M., Liebana, E., 2004. Characterization of pathogenic *Vibrio parahaemolyticus* isolates from clinical sources in Spain and comparison with Asian and North American pandemic isolates. J. Clin. Microbiol. 42, 4672–4678.
Martinez-Urtaza, J., Simental, L., Velasco, D., DePaola, A., Ishibashi, M., Nakaguchi, Y., Nishibuchi, M., Carrera-Flores, D., Rey-Alvarez, C., Pousa, A., 2005. Pandemic *Vibrio parahaemolyticus* O3:K6. Europe. Emerg. Infect. Dis. 11, 1319–1320.
Martinez-Urtaza, J., Lozano-Leon, A., Varela-Pet, J., Trinanes, J., Pazos, Y., Garcia-Martin, O., 2008. Environmental determinants of the occurrence and distribution of *Vibrio parahaemolyticus* in the Rias of Galicia, Spain. Appl. Environ. Microbiol. 74, 265–274.
Martinez-Urtaza, J., Baker-Austin, C., Jones, J.L., Newton, A.E., Gonzalez-Aviles, G.D., DePaola, A., 2013. Spread of pacific northwest *Vibrio parahaemolyticus* strain. N. Engl. J. Med. 369, 1573–1574.
McLaughlin, J.B., DePaola, A., Bopp, C.A., Martinek, K.A., Napolilli, N.P., Allison, C.G., Murray, S.L., Thompson, E.C., Bird, M.M., Middaugh, J.P., 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N. Engl. Jour. Med. 353, 1463–1470.
Nair, G.B., Ramamurthy, T., Bhattacharya, S.K., Dutta, B., Takeda, Y., Sack, D.A., 2007. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin. Microbiol. Rev. 20, 39–48.
Nandi, B., Nandy, R.K., Mukhopadhyay, S., Nair, G.B., Shimada, T., Ghose, A.C., 2000. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to gene of outer membrane protein OmpW. J. Clin. Microbiol. 38, 4145–4151.
Newton, A., Kendall, M., Vugia, D.J., Henao, O.L., Mahon, B.E., 2012. Increasing rates of vibriosis in the United States, 1996–2010: review of surveillance data from 2 systems. Clin. Infect. Dis. 54, S391–S395.
Nishibuchi, M., Kaper, J.B., 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infect. Immun. 63, 2093–2099.
Olive, D.M., Bean, P., 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. 37, 1661–1669.
Ottaviani, D., Leoni, F., Rocchegiani, E., Santarelli, S., Canonico, C., Masini, L., Ditrani, V., Carraturo, A., 2008. First clinical report of pandemic *Vibrio parahaemolyticus* O3:K6 infection in Italy. J. Clin. Microbiol. 46, 2144–2145.
Ottaviani, D., Leoni, F., Rocchegiani, E., Santarelli, S., Masini, L., Di Trani, V., Canonico, C., Pianetti, A., Tega, L., Carraturo, A., 2009. Prevalence and virulence properties of non-O1 non-O139 *Vibrio cholerae* strains from seafood and clinical samples collected in Italy. Int. J. Food Microbiol. 132, 47–53.
Ottaviani, D., Leoni, F., Rocchegiani, E., Canonico, C., Potenziani, S., Santarelli, S., Masini, L., Mioni, R., Carraturo, A., 2010. Prevalence, serotyping and molecular characterization of *Vibrio parahaemolyticus* in mussels from Italian growing areas, Adriatic Sea. Environ. Microbiol. Rep. 2, 192–197.
Ottaviani, D., Leoni, F., Rocchegiani, E., Mioni, R., Costa, A., Virgilio, S., Serracca, L., Bove, D., Canonico, C., Di Cesare, A., Masini, L., Potenziani, S., Caburlotto, G., Ghidini, V., Lleo, M.M., 2012a. An extensive investigation into the prevalence and the genetic and serological diversity of toxigenic *V. parahaemolyticus* in Italian marine coastal waters. Environ. Microbiol. 15, 1377–1386.
Ottaviani, D., Leoni, F., Serra, R., Serracca, L., Decastelli, L., Rocchegiani, E., Masini, L., Canonico, C., Talevi, G., Carraturo, A., 2012b. Nontoxigenic Vibrio parahaemolyticus strains causing acute gastroenteritis. J. Clin. Microbiol. 50, 4141–4143.
Panicker, G., Call, D.R., Krug, M.J., Bej, A.K., 2004. Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. Appl. Environ. Microbiol. 70, 7436–7444.
Paranjpye, R., Hamel, O.S., Stojanovski, A., Liermann, M., 2012. Genetic diversity of clinical and environmental *Vibrio parahaemolyticus* strains from the Pacific Northwest. Appl. Environ. Microbiol. 78, 8631–8638.
Passalacqua, P.L., Zavatta, E., Bignami, G., Serraino, A., Serratore, P., 2016. Occurrence of *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus* in the clam *Ruditapes philippinarum* (adams & reeve, 1850) from emilia romagna and Sardinia. Italy. Ital. J. Food Safety 5, 41–46.
Quilici, M.L., Robert-Pillot, A., Picart, J., Fournier, J.M., 2005. Pandemic *Vibrio parahaemolyticus* O3:K6 spread, France. Emerg. Infect. Dis. 11, 1148–1149.
Rao, B.M., Surendran, P.K., 2010. Genetic heterogeneity of non-O1 and non-O139 *Vibrio cholerae* isolates from shrimp aquaculture system: a comparison of RS-, REP- and ERIC-PCR fingerprinting approaches. Lett. Appl. Microbiol. 51, 65–74.
Robert-Pillot, A., Gueénole, A., Lesne, J., Delesmont, R., Fournier, J.M., Quilici, M.L., 2004. Occurrence of the tdh and trh in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. Int. J. Food Microbiol. 91, 319–325.
Rodriguez, J.M., Lopez-Romalde, S., Beaz, R., Alonso, M.C., Castro, D., Romalde, J.L., 2006. Molecular fingerprinting of *Vibrio tapetis* strains using three PCR-based methods: ERIC-PCR. REP-PCR and RAPD. Dis. Aquat. Org. 69, 175–183.
Rodriguez-Castro, A., Ansede-Bermejo, J., Blanco-Abad, V., Varela-Pet, J., Garcia-Martinez, O., Martinez-Urtaza, J., 2010. Prevalence and genetic diversity of pathogenic populations of *Vibrio parahaemolyticus* in coastal waters of Galicia, Spain. Environ. Microbiol. Rep. 2, 58–66.
Sawabe, T., Kita-Tsukamoto, K., Thompson, F.L., 2007. Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. J. Bacteriol. 189, 7932–7936.
Serracca, L., Battistini, R., Rossini, I., Prearo, M., Ottaviani, D., Leoni, F., Ercolini, C., 2011. *Vibrio virulence* genes in fishes collected from estuarine waters in Italy. Lett. Appl. Microbiol. 53, 403–408.
Sferlazzo, G., Meloni, D., Lamon, S., Marceddu, M., Mureddu, A., Consolati, S.G., Pisanu, M., Virgilio, S., 2018. Evaluation of short purification cycles in naturally contaminated Mediterranean mussels (*Mytilus galloprovincialis*) harvested in Sardinia (Italy). Food Microbiol. 74, 86–91.
Silva-Rubio, A., Acevedo, C., Magarinos, B., Jaureguiberry, B., Toranzo, A.E., Avendano-Herrera, R., 2008. Antigenic and molecular characterization of *Vibrio ordalii* strains isolated from Atlantic salmon *Salmon salar* in Chile. Dis. Aquat. Org. 79, 27–35.
Staley, C., Harwood, V.J., 2010. The use of genetic typing methods to discriminate among strains of vibrio cholerae, v. parahaemolyticus, and v. vulnificus. J. AOAC Int. 93 (5), 1553–1569.
Su, Y.C., Liu, C., 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. Food Microbiol. 24, 549–558.
Suffredini, E., Mioni, R., Mazzette, R., Bordin, P., Serratore, P., Fois, F., Piano, A., Cozzi, L., Croci, L., 2014. Detection and quantification of *Vibrio parahaemolyticus* in shellfish from Italian production areas. Int. J. Food Microbiol. 184, 14–20.
Takahashi, H., Hara-Kudo, Y., Miyasaka, J., Kamagai, S., Konuma, H., 2005. Development of a quantitative real-time polymerase chain reaction targeted to the toxR for detection of *Vibrio vulnificus*. J. Microbiol. Methods 61, 77–85.
Thompson, F.L., Gevers, D., Thompson, C.C., Dawyndt, P., Naser, S., Hoste, B., Munn, C.B., Swings, J., 2005. Phylogeny and molecular identification of vibrios on the basis

of multilocus sequence analysis. Appl. Environ. Microbiol. 71, 5107–5115.
Thongjun, J., Mittraparp-Arthorn, P., Yingkajorn, M., Kongreung, J., Nishibuchi, M., Vuddhakul, V., 2013. The trend of *Vibrio parahaemolyticus* infections in southern Thailand from 2006 to 2010. Trop. Med. Health 41, 151–156.
Turner, J.W., Paranjpye, R.N., Landis, E.D., Biryukov, S.V., Gonzalez-Escalona, N., Nilsson, W.B., Strom, M.S., 2013. Population structure of clinical and environmental *Vibrio parahaemolyticus* from the pacific northwest coast of the United States. PLoS One 8, e55726.
van Belkum, A., Struelens, M., de Visser, A., Verbrugh, H., Tibayrenc, M., 2001. Role of genomic typing in taxonomy, evolutionary genetics and microbial epidemiology. Clin. Microbiol. Rev. 14, 547–560.
Versalovic, J., Koeuth, T., Lupsky, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19, 6823–6831.
Versalovic, J., Schneither, M., De Bruijin, F.J., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. 5, 25–40.
Wagley, S., Koofhethile, K., Wing, J.B., Rangdale, R., 2008. Comparison of V. *parahaemolyticus* isolated from seafoods and cases of gastrointestinal disease in UK. Int. J. Environ. Health Res. 18, 283–293.
Wong, H.-C., Lin, C.-H., 2001. Evaluation of typing of *Vibrio parahaemolyticus* by three PCR methods using specific primers. J. Clin. Microbiol. 39, 4233–4240.
Wootipoom, N., Bhoopong, P., Pomwised, R., Nishibuchi, M., Ishibashi, M., Vuddhakul, V., 2007. A decrease in the proportion of infections by pandemic *Vibrio parahaemolyticus* in Hat Yai Hospital, southern Thailand. J. Med. Microbiol. 56, 1630–1638.
Xu, F., Ilyas, S., Hall, J.A., Jones, S.H., Cooper, V.S., Whistler, C.A., 2015. Genetic characterization of clinical and environmental *Vibrio parahaemolyticus* from the Northeast USA reveals emerging resident and non-indigenous pathogen lineages. Front. Microbiol. 6, 272.
Yoke-Kqueen, C., Teck-Ee, K., Son, R., Yoshitsugu, N., Mitsuaki, N., 2013. Molecular characterisation of Vibrio parahaemolyticus carrying tdh and trh genes using ERIC-, RAPD- and BOX-PCR on local Malaysia bloody clam and Lala. Int. Food Res. J. 20 (6), 3299–3305.
Zarei, M., Borujeni, M.P., Jamnejad, A., Khezrzadeh, M., 2012. Seasonal prevalence of Vibrio species in retail shrimps with an emphasis on *Vibrio parahaemolyticus*. Food Control 25, 107–109.
Zhang, X.H., Austin, B., 2005. Haemolysins in Vibrio species. J. Appl. Microbiol. 98, 1011–1019.
Zhang, L., Orth, K., 2013. Virulence determinants for *Vibrio parahaemolyticus* infection. Curr. Opin. Microbiol. 16, 70–77.