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Evaluation of different procedures for the optimized detection of *Vibrio parahaemolyticus* in mussels and environmental samples

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ABSTRACT

Vibrio parahaemolyticus is a marine bacterium with a worldwide distribution and is frequently associated with human outbreaks of infection. Detection and isolation of *V. parahaemolyticus* from natural sources is often problematical because of limitations in the analytical procedures. We evaluated a combination of conventional and molecular protocols previously described for the investigation of *V. parahaemolyticus*, with the aim of identifying the best procedures for improved detection of this organism in environmental matrixes. A total of 259 samples of zooplankton (103), mussels (48) and seawater (108) were investigated by an Absence–Presence method (A/P), whereas 118 samples of zooplankton (70) and mussels (48) were analyzed by the Most Probable Number (MPN) method. All samples were processed by a two-step enrichment procedure, firstly with APW broth and then with SPB as selective secondary broth. Detection of *V. parahaemolyticus* was by direct-PCR and by plate culture on TCBS and CHROMagar Vibrio, after sample enrichment in APW and SPB. With the A/P method, *V. parahaemolyticus* was detected in 23.6% samples by direct-PCR, whereas only 11.2% samples were positive with the plate culture method. With the MPN method, *V. parahaemolyticus* was detected in 54.2% and 27.1% of the samples by direct-PCR and plate culture respectively; this indicated the existence of 31% false negative results with the A/P method. No significant differences between the use of a single (APW) or two-step enrichment (APW+SPB) were observed by direct-PCR with A/P or MPN, although a significant higher presence of *V. parahaemolyticus* was detected by plate culture in both protocols with the two-step enrichment procedure. In conclusion, direct-PCR after sample enrichment in APW broth was the most successful method for detection of *V. parahaemolyticus* with the A/P procedure and enumeration by MPN. Better detection was obtained with MPN than with the A/P protocol. Conversely, the plate culture procedure showed better results with the two-step enrichment protocol in which CHROMagar Vibrio was used as the selective agar.

1. Introduction

Vibrio parahaemolyticus is a marine bacterium that occurs naturally in coastal waters worldwide and is a cause of gastroenteritis associated with the consumption of seafood. Only some populations of *V. parahaemolyticus* have enteropathogenic potential (Joseph et al., 1982), which is characteristically associated with the production of thermostable direct hemolysin (TDH), encoded by the *tdh* gene, and/or of thermostable direct hemolysin-related hemolysin (TRH), encoded by the *trh* gene. More than 90% of clinical *V. parahaemolyticus* isolates, but less than 1% of food or environmental strains, express TDH or contain the *tdh* gene (Honda et al., 1988; DePaola et al., 1990; Miyamoto et al., 1969; Tada et al., 1992).

Since *V. parahaemolyticus* was first detected in Japan in 1950 (Fujino et al., 1953), several protocols have been developed in which different sample diluents, enrichment broths and culture plates have been considered to detect and quantify *V. parahaemolyticus* in different samples (Beuchat, 1977; Donovan and van Netten, 1995; Ray et al., 1978; Twedt and Novelli, 1971). The most common conventional method for routine analyses includes a two-step protocol in which a single enrichment broth is used, followed by a culture on agar plate, characteristically TCBS agar (ISO 8914, 1990; U.S. Food and Drug Administration, 2004). Since its introduction, TCBS agar has been extensively used and to date is still the most commonly used selective medium for the isolation of Vibrio spp. from natural environments and clinical sources. Nevertheless, several limitations associated with the use of TCBS agar for the correct identification of *V. parahaemolyticus* colonies have been reported in recent years (Hara-Kudo et al., 2001, 2003; Martinez-Urtaza et al., 2008; Miwa et al., 2003; Su et al., 2005). On TCBS, *V. parahaemolyticus* shows similar morphology and characteristics as other Vibrio spp. (*V. vulnificus*, *V. mimicus*, *V. alginolyticus*, *V. fluvialis*), *Photobacterium spp*., *Chryseomonas spp*. or *Shewanella spp*. In addition to this lack of specificity, the limited selectivity of the medium allows for the overgrowth of other bacteria that predominate in the environment, such as in the case of *V. alginolyticus* in the Atlantic coast of Europe, thereby masking the presence of *V. parahaemolyticus* colonies and complicating the identification of Vibrio colonies

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Table 1
Target genes and oligonucleotide primer sequences for detection of total and pathogenic *Vibrio parahaemolyticus*

Primer	Sequence	Length (bp)	Amplicon size (bp)	Target gene	Original reference
toxR (Forward)	5'- GTCTTCTGACGCAATCGTTG - 3'	20	368	*Vp-tox R*	Kim et al. (1999)
toxR (Reverse)	5'- ATACGACTGGTTGCTGTCATG - 3'	21			
tdh (Forward)	5'- CCACTACCACTCTCATATGC - 3'	20	251	*tdh*	Tada et al. (1992)
tdh (Reverse)	5'- GGTACTAAATGGCTGACATC - 3'	20			
trh (Forward)	5'- GGCTCAAAATGGTTAAGCG - 3'	19	250	*trh*	Tada et al. (1992)
trh (Reverse)	5'- CATTTCCGCTCTCATATGC - 3'	19			

(Martinez-Urtaza et al., 2008). A new chromogenic agar (CHROMagar Vibrio, Paris, France) has recently been developed specifically to differentiate *V. parahaemolyticus* from other bacteria by use of a chromogenic substrate, rather than the sugar fermentation used in traditional growth media such as TCBS (Hara-Kudo et al., 2001, 2003). Furthermore, to avoid interference caused by the overgrowth of other bacteria, some modified protocols for the detection of *V. parahaemolyticus* have proposed the addition of a selective enrichment step in the procedure, prior to plating onto the selective agar (Donovan and van Netten, 1995; Hara-Kudo et al., 2001, 2003).

Despite the improvements in conventional plate culture methods, the protocols are time-consuming and laborious, and require more than 5 days for confirmatory results. In addition, conventional methods do not discriminate pathogenic specimens from *V. parahaemolyticus* populations. A considerable advance in the improvement of these procedural limitations has been achieved in recent years through the development of molecular techniques for the identification and characterization of Vibrio species (Blackstone et al., 2003; Elliot et al., 1995; Miwa et al., 2003; Raghunath et al., 2007a,b; Ward and Bej, 2006). Several new methods for gene-identification based on PCR or DNA hybridization procedures have been designed. A DNA colony hybridization technique that targets the *V. parahaemolyticus*-specific gene *tlh* and the pathogenic gene *tdh* has been developed to quantify total and pathogenic *V. parahaemolyticus* with alkaline phosphatase probes (Ellison et al., 2001; McCarthy et al., 1999, 2000). This protocol greatly reduces the time and effort required for sample analysis. However, this colony hybridization protocol is based on direct plating onto a non-selective agar, which allows the growth of background microflora in samples and may result in the target organism being masked (Kaufman et al., 2004). Advances in PCR technologies have recently contributed to improving the detection of *V. parahaemolyticus* in environmental samples, thereby providing higher speed, reliability and simplicity in analytical procedures (Bej and Mahbubani, 1992; Swaminathan and Feng, 1994). Nevertheless, the need for abundant and expensive materials and equipment, as well as highly-qualified technicians, has limited the use of these procedures in routine analysis.

Another factor affecting the extensive use of PCR procedures is related to the absence of a gold standard protocol for the detection of *V. parahaemolyticus*, which has been difficult, due to the limited information regarding the most suitable culture media for an enhanced growth of *V. parahaemolyticus*. Here we evaluated the efficiency of conventional and molecular methods with different combinations of broths and agar plates for analysis of *V. parahaemolyticus* in environmental samples, with the aim of identifying the best protocol for optimal detection of the bacterium.

2. Materials and methods

2.1. Sample collection

The study included the analysis of 259 environmental samples of different origin: 108 samples of seawater, 103 of zooplankton and 48 of mussels (*Mytilus galloprovincialis*). All samples were collected during the sampling program of an ongoing study of *V. parahaemolyticus* in coastal areas of Galicia (Spain) carried out from July of 2005 to June of 2006. Zooplankton samples were collected by oblique tow, with a 200 μm mesh net and were placed in sterile bottles and diluted with sterile seawater. Seawater samples comprised of 150 ml of water was obtained from different depths with Niskin bottles, and transferred to sterile plastic containers. Mussel samples of 1 kg each were collected from the ropes of rafts used for mussel cultivation and were transferred to sterile plastic bags for transportation.

All samples were transported in portable coolers at ambient temperature and were analyzed immediately on arrival to the laboratory.

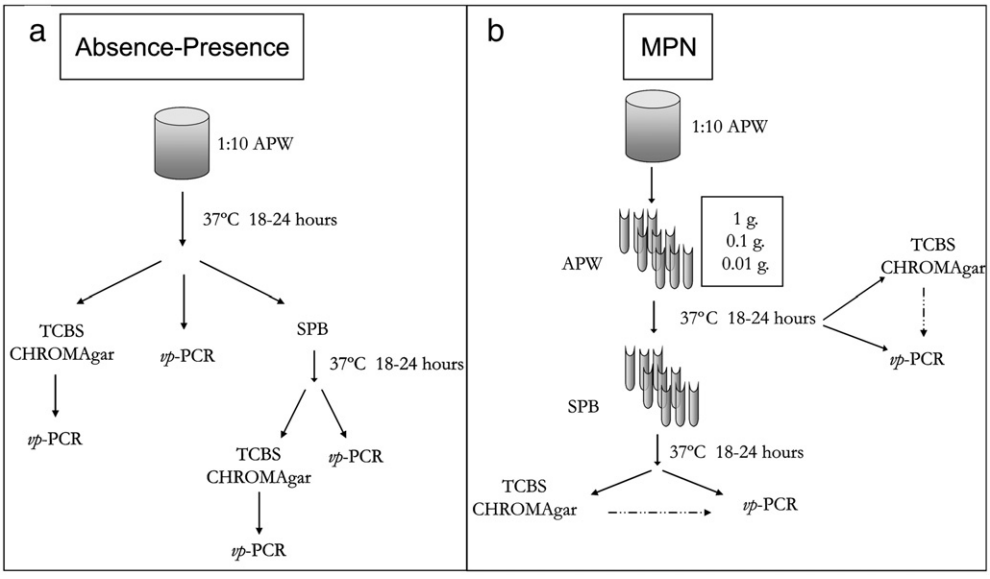

Fig. 1. Diagram illustrating the Absence–Presence (a) and MPN (b) procedures employed in the study.

Table 2
Results obtained from the analysis of water, zooplankton and mussel samples by use of the Absence–Presence and MPN methods for detection of total and pathogenic *V. parahaemolyticus*, via direct-PCR and conventional plate method, with one (APW) or two step enrichment (APW + SPB)

Method		Type of samples	No. samples	Gene					
				toxR		*tdh*		*trh*	
				APW[a]	SPB	APW	SPB	APW	SPB
Absence–Presence	Plate culture	Zooplankton	103	6 (5.8%)	15 (14.6%)	0	0	3 (2.9%)	4 (3.9%)
		Mussel	48	1 (2.1%)	8 (16.7%)	0	1 (2.1%)	0	1 (2.1%)
		Water	108	3 (2.8%)	6 (5.6%)	0	0	1 (0.9%)	0
		Total	259	10 (3.9%)	29 (11.2%)	0	1 (0.4%)	4 (1.5%)	5 (1.9%)
	Direct-PCR	Zooplankton	103	39 (37.9%)	29 (28.2%)	3 (2.9%)	1 (1%)	5 (4.9%)	5 (4.9%)
		Mussel	48	12 (25%)	16 (33.3%)	0	0	1 (2.1%)	3 (6.3%)
		Water	108	10 (9.3%)	11 (10.2%)	0	0	1 (0.9%)	2 (1.9%)
		Total	259	61 (23.6%)	56 (21.6%)	3 (1.2%)	1 (0.4%)	7 (2.7%)	10 (3.9%)
MPN	Plate culture	Zooplankton	70	14 (20%)	26 (37.1%)	2 (2.9%)	2 (2.9%)	5 (7.1%)	8 (11.4%)
		Mussel	48	5 (10.4%)	6 (12.5%)	0	0	0	1 (2.1%)
		Total	118	19 (16.1%)	32 (27.1%)	2 (1.7%)	2 (1.7%)	5 (4.2%)	9 (7.6%)
	Direct-PCR	Zooplankton	70	42 (60%)	44 (62.9%)	6 (8.6%)	1 (1.4%)	15 (21.4%)	16 (22.9%)
		Mussel	48	22 (45.8%)	19 (39.6%)	1 (2.1%)	2 (4.2%)	0	1 (2.1%)
		Total	118	64 (54.2%)	63 (53.4%)	7 (5.9%)	3 (2.5%)	15 (12.7%)	17 (14.4%)

[a]Positive samples (%).

2.2. Sample preparation

In the laboratory, mussels were immediately removed from the bags, washed and scrubbed under running potable water to remove debris and attached algae; dead mussels or those with broken shells were discarded. Approximately 20 to 25 specimens were opened aseptically with a sterilized scalpel. Meat and shell liquid were collected in a sterile jar, cut with scissors and mixed thoroughly. Fifty grams of liquid and meat were transferred into sterile plastic bags with 450 ml of alkaline peptone water (APW; 1% [wt/vol] peptone, 1% [wt/vol] sodium chloride; pH 8.2) to obtain a 1:10 dilution and subjected to further homogenization for 60 s using a stomacher.

All the collected volume (150 ml) of each water sample was concentrated by filtration through a 0.45 µm-pore-size filter (Sartorius, Goettingen, Germany) with a vacuum pump, and filters were subsequently placed in 225 ml of APW.

Zooplankton samples were divided into three fractions (>200 µm, <200 µm and >50 µm, and <50 µm) by filtration through a 200 µm-mesh and 50 µm-mesh. Zooplankton bigger than 200 µm (Mesozooplankton) were retained into the 200 µm filter, washed with sterile water, weighed, diluted 1:10 in APW broth and analyzed using MPN method; zooplankton smaller than 200 µm passed through the 200 µm-mesh and was retained into the 50 µm, obtaining small amounts of this fraction that was transferred to 250 ml of APW without quantification and, hence, only subjected to Absence–Presence investigation; finally, zooplankton smaller than 50 µm passed through the 200 µm-mesh and 50 µm-mesh was recovered and weighed and processed by MPN.

2.3. Analysis of V. parahaemolyticus

All the 259 samples investigated in the present study (108 of water, 103 of zooplankton, and 48 of mussels) were analyzed by the Absence–Presence method but only the mussels samples (48) and the zooplankton fractions >200 µm (35) and <50 µm (35) were also analyzed in parallel by a standard quantitative method involving the most probable number technique (MPN) (Fig. 1a and b).

2.3.1. Absence–Presence method (A/P)

The qualitative method was performed on the basis of the ISO 8914 standard method (ISO 8914, 1990) modified according to the procedure of Hara-Kudo et al. (2001, 2003), as adapted for this study (Fig. 1a). Samples in APW broth were homogenized for 60 s with a stomacher (IUL Instruments) and incubated at 37 °C for 16–18 h. After incubation, 0.1 ml of APW broth was transferred to 10 ml salt polymyxin B broth (SPB; 1% [wt/vol] Peptone, 3% [wt/vol] Yeast extract, 2% [wt/vol] NaCl and 100,000 IU Polymyxin B, pH 7.4) and incubated at 37 °C for 18 to 24 h. One-ml aliquots of APW and SPB enrichment broths showing growth were analyzed by PCR and were additionally plated onto two thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates (Oxoid, Hampshire, United Kingdom) and a single CHROMagar Vibrio plate (CHROMagar Microbiology, Paris, France), and incubated for 16–18 h at 37 °C.

Table 3
Maximum and mean MPN values (MPN/100 g) for the different combinations of broths and protocols evaluated in the study

Method	Broth	Gene	Source					
			Zooplankton + Mussel[a]		Zooplankton[a]		Mussel[a]	
			Maximum	Mean	Maximum	Mean	Maximum	Mean
Plate culture	APW	*toxR*	430	34.99	430	49.74	210	23.19
		tdh	110	16.31	110	17.20	15	15
		trh	110	18.72	110	21.27	15	15
	SPB	*toxR*	11,000	173.78	350	66.23	110	22.3
		tdh	74	15.68	74	16.14	15	15
		trh	280	19.64	280	22.51	36	15.44
Direct-PCR	APW	*toxR*	>11,000	1782.13	>11,000	3134.08	>11,000	997.35
		tdh	>11,000	111.51	>11,000	176.84	74	16.23
		trh	>11,000	122.37	>11,000	196.0	15	15
	SPB	*toxR*	>11,000	883.74	>11,000	1456.09	>11,000	556.58
		tdh	74	15.86	36	15.30	74	16.67
		trh	4600	126.42	4600	202.51	36	15.44

[a] MPN/100 g.

Table 4
Distribution of MPN results according to count levels. Level 0: <30 MPN/100 g; level 1: 30–92 MPN/100 g; level 2: 110–930 MPN/100 g; level 3: 2100–11,000 MPN/100 g; level 4:>11,000 MNP/100 g

Method	Broth	Gene	Level 0[a]			Level 1[a]			Level 2[a]			Level 3[a]			Level 4[a]		
			Zooplankton	Mussel	Total	Zooplankton	Mussel	Total	Zooplankton	Mussel	Total	Zooplankton	Mussel	Total	Zooplankton	Mussel	Total
Plate culture	APW	*toxR*	77.15	89.57	82.2	8.57	8.33	8.5	14.28	2.1	9.3	0	0	0	0	0	0
		tdh	97.2	100	98.4	1.4	0	0.8	1.4	0	0.8	0	0	0	0	0	0
		trh	92.9	100	95.8	1.4	0	0.8	5.7	0	3.4	0	0	0	0	0	0
	SPB	*toxR*	61.47	87.47	72.9	15.71	8.33	12.7	21.42	4.2	13.6	1.4	0	0.8	0	0	0
		tdh	97.15	100	98.3	2.85	0	1.7	0	0	0	0	0	0	0	0	0
		trh	88.6	97.9	97.5	10	2.1	1.7	1.4	0	0.8	0	0	0	0	0	0
Direct-PCR	APW	*toxR*	40.1	54.25	45.9	11.4	16.6	13.5	20	16.6	18.6	11.4	6.25	9.3	17.1	6.3	12.7
		tdh	91.49	97.9	94.17	5.71	2.1	4.23	1.4	0	0.8	0	0	0	1.4	0	0.8
		trh	78.61	100	87.34	8.57	0	5.08	11.42	0	6.78	0	0	0	1.4	0	0.8
	SPB	*toxR*	37.18	60.41	46.7	25.7	12.5	20.3	22.85	20.83	22	8.57	4.16	6.8	5.7	2.1	4.2
		tdh	98.6	95.83	97.46	1.4	4.17	2.54	0	0	0	0	0	0	0	0	0
		trh	77.16	97.9	85.6	11.42	2.1	7.63	7.14	0	4.23	4.28	0	2.54	0	0	0

[a]% samples.

The consecutive streaking of two TCBS plates was used as a strategy to obtain enhanced isolation of Vibrio species other than *V. alginolyticus* on the plate with the lowest concentrate of inoculum, and to minimize the possibility of false negative assays due to overgrowth of *V. alginolyticus*, according to previous observations (Martinez-Urtaza et al., 2008). After overnight incubation, between three and five presumptive colonies of *V. parahaemolyticus* growing on TCBS (green) and CHROMagar Vibrio (violet) plates were randomly selected and cultured in TSA containing 2% NaCl (Difco). TSA plates were incubated at 37 °C for 24 h and identification of the colonies was confirmed by API20E strips (BioMérieux, Marcy-l´Etoile, France) and polymerase chain reaction (PCR) analysis.

2.3.2. Most Probable Method (MPN)

V. parahaemolyticus was quantified by the MPN technique following the Bacteriological Analytical Manual procedure (Elliot et al., 1995), with the modifications described by Hara-Kudo et al. (2001, 2003) adapted for this study (Fig. 1b). Homogenized 1:10 APW dilutions of zooplankton and mussel samples were used in the three-tube MPN technique. A total of 10 ml of the 1:10 APW dilution (containing 1 g of sample), and 1 ml of 1:10 and 1:100 dilutions were inoculated in the three tubes of three MPN series and were incubated at 37 °C for 18 to 24 h. Aliquots of 0.1 ml of APW broths were transferred to tubes containing 10 ml of SPB and incubated at 37 °C for 18 to 24 h. After incubation, the APW and SPB broths were analyzed simultaneously by PCR and plated onto two TCBS plates and a single CHROMagar Vibrio plate. The plates were incubated overnight at 37 °C, and between three and five *V. parahaemolyticus* presumptive colonies were selected at random from each plate, cultured in TSA containing 2% NaCl (Difco) and incubated at 37 °C for 24 h before confirmation of colony identification with API20E strips and PCR analysis.

2.4. PCR analysis

2.4.1. Genomic DNA extraction

Aliquots of APW and SPB broths were used for DNA extraction. One ml of broth was centrifuged at 2000 rpm for 2 min to discard particles in suspension. The supernatant was transferred to a new tube and centrifuged again at 9000 ×*g* for 10 min. The supernatant was discarded and the resultant pellet was resuspended in 300 µl 1xTE buffer (10 mM Tris pH8.0, 1 mM EDTA) and centrifuged again at 9000 ×*g* for 7 min. After the supernatant was discarded, the pellet was resuspended with 300 µl of 5% Chelex (BioRad, Hercules, California) in sterile water and heated at 56 °C for 20 min. In order to lyse the cells, the suspension was boiled at 100 °C for 10 min and immediately placed on ice for 2 min. The lysate was centrifuged at 10,000 ×*g* and 4 °C for 5 min, and the supernatant containing the DNA was transferred to a new tube and stored at −20 °C until its use as a template for PCR analysis.

For DNA extraction of isolates, a loop of pure culture was transferred to 5 ml of Luria-Bertani broth with 2% NaCl incubated overnight at 37 °C in a shaking water bath. One ml of culture broth was transferred to a 1.5 ml tube and centrifuged at 9000 ×*g* for 7 min. The resulting pellet was resuspended in 300 µl of 1×TE buffer and cell suspensions were lysed by boiling for 10 min. The lysate was centrifuged at 10,000 ×*g* and 4 °C for 5 min, the supernatant was transferred to a new tube and stored at −20 °C until use.

DNA quantification was performed diluting DNA 1:10 in nuclease free water (SIGMA) and absorbance was measured at 260 nm with a Bio-Photometer (Eppendorf, Hamburg, Germany).

2.4.2. PCR detection

The presence of *V. parahaemolyticus* in broths and confirmation of the presumptive isolates was investigated by use of the *V. parahaemolyticus* specific gene Vp-*toxR*, according to the PCR protocol described by Kim et al. (1999) (Table 1).

Presence of virulence related genes *tdh* and *trh* in broth and isolates was determined using independent PCR assays for each gene, according to Tada et al. (1992) (Table 1). The PCR reactions were carried out in a PTC 200 thermocycler (MJ Research, South San Francisco, California). All PCR products were checked by electrophoresis on 1.6% agarose gels (Agarose type II, Sigma-Aldrich, USA) stained with ethidium bromide (0.5 µg/ml; Sigma) and photographed with an Alpha Innotech 2200 UV transilluminator and processed with an Alphaimager 2200 gel documentation system (Alpha Innotech, San Leandro, California). Strains without amplification of Vp-*toxR* by PCR were considered negative for *V. parahaemolyticus* and were not subjected to further analysis for *tdh* and *trh* genes.

The reference strains AQ4037 (*trh*$^+$, *tdh*$^-$), ATCC43996 (*tdh*$^+$, *trh*$^-$) and VP81 (pandemic strain, *trh*$^+$, *tdh*$^-$) were used as positive controls in all biochemical tests and PCR assays.

2.5. Statistical analysis

Pairs of results obtained from the different methods were compared by use of the Wilcoxon non-parametric test. All statistical

Table 5
Comparative results for detection of *V. parahaemolyticus*, according to the different protocols and combinations of culture media used

Protocol	AP−/MPN−	AP−/MPN+	AP+/MPN−	AP+/MPN+	Total
APW direct-PCR	49	32	5	32	118
SPB direct-PCR	49	33	6	30	118
APW plate culture	97	15	2	4	118
SPB plate culture	80	18	6	14	118
TOTAL	275	98	19	80	472

Table 6
Presumptive and confirmed results of positive samples for presence of *V. parahaemolyticus* obtained with TCBS agar and CHROMagar

Method	Agar plate	No. samples	APW		SPB	
			Presumptive (%)	Confirmed (%)	Presumptive (%)	Confirmed (%)
Absence–Presence	TCBS	309	86 (27.8%)	7 (8.13%)	95 (30.7%)	8 (8.4%)
	CHROMagar	309	55 (17.8%)	12 (21.8%)	95 (30.7%)	26 (27.4%)
MPN	TCBS	126	81 (64.3%)	14 (17.3%)	84 (66.7%)	20 (35.7%)
	CHROMagar	126	68 (54%)	25 (36.8%)	65 (51.6%)	22 (33.8%)

analyses were carried out with SPSS version 14.0.1 (SPSS Inc.) and the level of significance was set at $P<0.05$.

3. Results

3.1. Absence–Presence method

A total of 103 samples of zooplankton, 48 of mussels and 108 of seawater were examined by the absence-presence method (A/P). Detection of *V. parahaemolyticus* by the use of plate culture was significantly lower than by the direct-PCR method. *V. parahaemolyticus* was detected in 61 samples (23.6%) by direct-PCR, whereas only 29 samples (11.2%) were positive by plate culture (Table 2). No significant differences between single (APW) (23.6%) and the two-step enrichment (APW+SPB) (21.6%) were observed by use of direct-PCR. By contrast, a significantly higher presence of *V. parahaemolyticus* was detected by plate culture and the two-step enrichment process (11.2%) than with enrichment only in APW broth (3.9%), using both TCBS and CHROMAgar plates.

Similar differences between the methods were observed in the three matrixes evaluated (Table 2). Higher presence of *V. parahaemolyticus* were detected by direct-PCR, with 37.9, 33.3 and 10.2% positive samples of zooplankton, mussels and seawater, respectively, in contrast to the 14.6, 16.7 and 5.6% positive samples obtained by plate culture method using both TCBS and CHROMAgar plates (Table 2). With direct-PCR, only zooplankton samples showed a significantly higher presence of *V. parahaemolyticus* with single enrichment, whereas no differences were observed in mussels and seawater. A higher presence of *V. parahaemolyticus* was detected in the three matrixes by inclusion of the two-step enrichment with the plate culture procedure.

There were no significant differences in the detection of *tdh* or *trh* positive *V. parahaemolyticus* between the plate culture method, direct-PCR, and enrichment broths in the different matrixes analyzed, although the result may have been strongly influenced by the small number of positive samples obtained in the study (Table 2).

3.2. MPN method

One hundred and eighteen samples (70 samples of zooplankton and 48 samples of mussel) were analyzed by the MPN method.

Direct-PCR showed higher counts of *V. parahaemolyticus* than the plate culture method, and reached maximum MPN values (>11,000 MPN/100 g) in 15 samples (12.7%), and with a mean of 1782.127 MPN/100 g. Conversely, the plate culture method only reached maximum values of 11,000 MPN/100 g in 0.8% samples and showed an average of 173.78 MPN/100 g (Tables 3 and 4).

V. parahaemolyticus counts obtained by plate culture plus two-step enrichment were significantly higher (maximum 11,000 MPN/100 g and mean 173.78 MPN/100 g) than those obtained by single culture in APW (maximum 430 MPN/100 g and mean 34.99 MPN/100 g) (Table 3). By contrast, the mean values obtained by direct-PCR and APW were twice those obtained by inclusion of the two-step enrichment process (1782.13 MPN/100 g and 883.74 MPN/100 g respectively). Similar differences between methods were observed in the two matrixes evaluated (zooplankton and mussels) (Table 3).

Enumeration of pathogenic *V. parahaemolyticus* with the MPN method provided significantly higher maximum counts for both *trh* and *tdh* genes by direct-PCR plus single-step enrichment (Table 3).

3.3. Comparison of V. parahaemolyticus detection by A/P and MPN methods

Results from MPN counts of zooplankton and mussels samples were categorized as qualitative values (positive and negative) and compared with the results of A/P method of those samples which were analyzed by the two methods simultaneously (118) to evaluate the efficiency and sensitivity of each method for detection of *V. parahaemolyticus*.

V. parahaemolyticus was more efficiently identified with the same combination of procedures than that observed by A/P method, although MPN technique yielded more than twice the number of positive samples than the A/P method in most of the procedural variations tested (Table 2). *V. parahaemolyticus* was detected by MPN and direct-PCR in 54.2% and 53.4% samples respectively with inclusion of single (APW) and two-step enrichment (APW+SPB), whereas by A/P method were obtained 23.6% and 21.6% of samples as positive. Similar differences were also observed with the plate culture method, with 16.1% and 27.1% positive samples detected by MPN plus APW and SPB respectively, in contrast to 3.9% and 11.2% obtained by A/P method. Related differences in sensitivity of detection for both methods were observed in all the matrixes evaluated (Table 2). The MPN method was also observed to be more efficient for the detection of pathogenic *V. parahaemolyticus*, with higher values of presence of *tdh* and *trh* positive *V. parahaemolyticus* detected in all cases.

In order to compare the efficiency of the different combinations of procedures in detecting *V. parahaemolyticus*, 472 sample results obtained by the different procedures were evaluated and results are shown in Table 5. The A/P and MPN methods yielded different results in 117 samples. *V. parahaemolyticus* was found in 98 samples that were negative by the A/P method, in contrast with the 19 positive results obtained with A/P, which were negative by the MPN method. The highest number of false negatives yielded by the A/P method was observed by direct-PCR (32 and 33 cases).

Table 7
Number of presumptive and confirmed *V. parahaemolyticus* isolates obtained with TCBS agar and CHROMagar through the study using A/P and MPN methods

Agar plate	APW		SPB		Total	
	Presumptive isolates	Confirmed isolates (%)	Presumptive isolates	Confirmed isolates (%)	Presumptive isolates	Confirmed isolates (%)
TCBS	557	53 (9.51%)	692	72 (10.4%)	1249	125 (10%)
CHROMagar	456	110 (24.12%)	608	185 (30.43%)	1064	295 (27.72%)

3.4. Comparison of TCBS agar and CHROMagar Vibrio

Of 309 samples analyzed for presence of *V. parahaemolyticus* by the A/P method with TCBS agar, 86 (27.8%) for APW and 95 (30.7%) for SPB showed presence of presumptive *V. parahaemolyticus* colonies on plates and were analyzed further to confirm the identification (Table 6). In only 7 (8.13%) samples enriched in APW and 8 (8.4%) enriched in SPB, the presumptive colonies were confirmed as *V. parahaemolyticus*. By contrast, the analysis of the same 309 samples with CHROMagar Vibrio revealed the presence of presumptive *V. parahaemolyticus* colonies in 55 (17.8%) samples enriched in APW broth and 95 (30.7%) samples enriched in SPB, of which 12 (21.8%) and 26 (27.4%) samples respectively were confirmed as *V. parahaemolyticus*.

With the MPN method, 126 samples were analyzed for the presence of *V. parahaemolyticus* with both TCBS and CHROMagar Vibrio (Table 6). With TCBS agar presumptive colonies were identified in 81 samples (64.3%) enriched in APW and 84 (66.7%) enriched in SPB, although only 14 (17.3%) and 20 (35.7%) samples were finally confirmed for presence of *V. parahaemolyticus*. With CHROMagar Vibrio plates, 68 samples (54%) enriched in APW and 65 (51.6%) enriched in SBP showed growth of presumptive *V. parahaemolyticus* colonies, with a final confirmed result of 25 (36.8%) and 22 (33.8%) positive samples in APW and SPB respectively.

Of all of the isolates investigated, 1249 and 1064 presumptive *V. parahaemolyticus* were obtained respectively from TCBS agar and CHROMagar Vibrio throughout the study (Table 7). Only 125 colonies on TCBS agar (10%) were confirmed as *V. parahaemolyticus*, in contrast with the 295 (27.72%) confirmed on CHROMagar Vibrio, which proved to be more sensitive and efficient for the isolation and identification of *V. parahaemolyticus*.

The purple strains isolated from CHROMagar Vibrio and identified as non-*Vibrio parahaemolyticus* were assigned in order of frequency to the following groups: *Aeromonas hydrophila*, *Vibrio fluvialis*, *Providenza rettgeri*, *Escherichia fergusonii*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. On TCBS agar, the green strains finally confirmed as non-*Vibrio parahaemolyticus* were identified as *Providenza rettgeri*, *Proteus mirabilis*, *Proteus vulgaris*, *Vibrio alginolyticus*, *Morganella morganii* and *Aeromonas hydrophila*.

4. Discussion

The growing importance of *V. parahaemolyticus* infections on a global scale has promoted the implementation of improved analytical procedures for the detection and enumeration of this pathogen in its natural environment. Conventional methods based on the use of culture plate media have been recognized to present serious limitations for analysis of *V. parahaemolyticus* owing to the overgrowth of other natural bacterial flora present in coastal environments (Martinez-Urtaza et al., 2008; Miwa et al., 2003), and to other problems associated with the limited cultivability of Vibrio under adverse conditions. Molecular methods have shown to be less time-consuming and more sensitive for *V. parahaemolyticus* detection in marine samples and the use of PCR for *V. parahaemolyticus* has become more common in recent years, mainly for research purposes. However, the need to obtain bacterial specimens for further characterization obliges the use of conventional culture methods to complement PCR procedures. Hence, both conventional and molecular methods should be improved to reduce their limitations and for their complementary use in routine analyses.

Many microbiological laboratories involved the quality control of seafood employ a single qualitative method to determine the presence of *Vibrio parahaemolyticus* in samples. The Absence–Presence method is probably the most extensively used procedure worldwide because of its simplicity, rapid results and low costs, especially in many developing countries in tropical areas where Vibrio diseases are endemic and where support for microbiological controls is limited. The results of the present study revealed that the use of the Absence–Presence method alone is not recommendable for routine and official controls because of the high number of false negative results obtained. The use of the ISO standard method (International Organization for Standardization (ISO), 1990 International Organization for Standardization (ISO), 1990. General guidance for the detection of *Vibrio parahaemolyticus*. ISO 8914:1990(E), Geneva, Switzerland. ISO 8914, 1990) to investigate the presence of *V. parahaemolyticus* failed to detect the presence of this organism when compared to traditional MPN technique, which has shown high sensitivity for *V. parahaemolyticus* detection with both plate culture and direct-PCR, independent of the enrichment broth used, with the additional advantage of providing quantification of the number of bacteria in the samples. MPN procedure consists of 9 tube test with three replicates of three sample dilutions (1:10, 1:100 and 1:1000). By contrast, Absence–Presence method consists of an unique tube with an enrichment of a 1:10 dilution of the sample in APW. The use three replicas of the most concentrated enrichment with two additional series of more diluted samples, results in a higher detection of presence of *V. parahaemolyticus* than the use of a single tube in the Absence–Presence procedure. If we consider the detection of *V. parahaemolyticus* by MPN in any tube as positive for the presence of this organism, we conclude that the Absence–Presence method failed to detect *V. parahaemolyticus* in 18% and 33% of the samples when culture plate and PCR were respectively employed in samples that were positive for presence of *V. parahaemolyticus* by MPN. Most of the positive MPN tubes for *V. parahaemolyticus* belonged to the two more diluted series, whereas many tubes of the most concentrated MPN series (1:10) showed a high incidence of false negatives (negatives in the 1:10 dilution and positive in the 1:100 and 1:1000 series). The presence of these false negative in the most concentrated series coincide with the false negative results detected using the Absence–Presence procedure, since both procedures consist of the incubations of 1/10 dilution of the sample in APW. However, the principle inconvenience of the MPN method is that the time and cost of analysis are nine times greater than with A/P method.

The results of the present study clearly indicate that the use of direct-PCR from culture broths was more successful in detecting *V. parahaemolyticus* than the plate culture method. Direct-PCR enabled detection of *V. parahaemolyticus* in at least double the number of samples than the plate culture method, and also provided higher values of MPN per 100 g. These results are similar to those of previous studies in which conventional microbiological culture methods were compared with PCR for the detection of *V. parahaemolyticus* in environmental samples (Bej et al., 1999; Bilung et al., 2005; Miwa et al., 2003, 2006).

The inclusion of a second selective enrichment step with SPB in both the A/P and MPN procedures did not provide any additional advantage for the detection of *V. parahaemolyticus* with direct-PCR relative to the use of APW only. Additionally, the protocol that included a single enrichment step in APW enabled us to obtain higher numbers of *V. parahaemolyticus*. By contrast, the protocol including the two-step enrichment yielded higher values for presence and counts than by plate culture. *Vibrio parahaemolyticus* was more readily detectable on plates when the procedures included a second selective enrichment in SPB with both Absence–Presence method and the MPN method. *V. parahaemolyticus* shows resistance to some antibiotics such as polymyxin B, the selective agent used in the SPB because of its inhibitory effect on other Vibrio spp. (Donovan and van Netten, 1995; Hara-Kudo et al., 2001). The use of a second selective step in the procedure reduces the overgrowth of other competitive flora, such as *V. alginolyticus*, dominant in the marine environment of the Atlantic coast of Europe (Martinez-Urtaza et al., 2008) and allows better growth of *V. parahaemolyticus* colonies on plates, resulting in improved identification and final results. However, the use of SPB may have a contrary effect on PCR results and the presence of

polymyxin B may cause the inhibition of PCR reactions, as reflected by the lower values of *V. parahaemolyticus* counts obtained with this broth.

Another common problem observed by use of direct-PCR with the MPN technique was the difficulty in detecting *V. parahaemolyticus* in tube-series with greater amounts of inocula from highly contaminated samples (samples that yielded positive results in the three-series tubes). Several studies have shown different causes of inhibition of PCR reactions associated with food or environmental samples (Blackstone et al., 2003; Hara-Kudo et al., 2003; Kaufman et al., 2004). Some components of food and culture media, such as fats and other organic compounds, have been reported to interfere or inhibit the PCR (Rossen et al., 1992). Large amounts of DNA template in the PCR reaction mix have also been identified as inhibitory for PCR reactions (Malorny et al., 2004). Although we could not evaluate the presence of chemical components as potential inhibitors in the present study, we included a chelating resin in the DNA-purification step (Chelex) to minimize the potential inhibitory effect associated with the presence of chemicals in samples (Walsh et al., 1991). In addition, dilution of the DNA extractions to reduce the concentration of potential inhibitory substances in the PCR reaction tubes was not advantageous in terms of improving gene detection. The evaluation of the concentration of DNA in each of the different MPN-tubes did not differ between the tubes in the different inoculum-series, and the presence of elevated amounts of DNA was therefore discounted as the main cause of inhibition. One possible solution to reducing inhibitions in PCR reactions may be the use of different systems of purifying the DNA during extraction, such as silica membrane and the glass fibre methods (Hara-Kudo et al., 2003). However, these are time-consuming and greatly increase the cost of analysis, which implies a serious constraint to extending their use in routine analysis. Further investigations are necessary to clarify this point, since this may imply serious procedural limitations.

The main obstacle for detection and isolation of *V. parahaemolyticus* from plate culture was the overgrowth of many colonies of Vibrio spp. other than *V. parahaemolyticus*, such as *V. alginolyticus*, and the high levels of other background microflora (Kaufman et al., 2004). The dominance of *V. alginolyticus* is especially high along the Atlantic coast of Europe and limits the detection of *V. parahaemolyticus*, which is less frequent and presents a slow growth rate relative to *Vibrio alginolyticus,* its main competitor on plates (Miwa et al., 2003; Martinez-Urtaza et al., 2008). The inclusion of a chromogenic agar such as CHROMagar Vibrio enhanced the isolation of presumptive colonies of *V. parahaemolyticus*, mainly because of the easy identification of mauve-coloured colonies such as *V. parahaemolyticus*, which together with the reduction in the presence of competitive flora on plates, results in improved sensitivity of plate culture methods. These results identified CHROMagar Vibrio as the most suitable selective medium for the isolation of *V. parahaemolyticus* from natural environments, similar to those previously reported by other authors (Hara-Kudo et al., 2001, 2003; Bilung et al., 2005).

Unfortunately, the low numbers of pathogenic strains present in the samples evaluated seriously restricts the conclusions that can be reached regarding the best method of detecting *tdh*-positive and *trh*-positive *V. parahaemolyticus*, although a preliminary evaluation can be made. The best results were obtained with the same combinations of protocols and media described for total *V. parahaemolyticus*, with more efficient detection of *tdh* and *trh*-positive *V. parahaemolyticus* by the use of direct-PCR and enrichment in APW broth, and the two-step protocol involving enrichment in SPB as the best procedure for the isolation of pathogenic strains on plates with the conventional culture method, similar to the results described by Hara-Kudo et al. (2001).

In summary, the results of the present study identified direct-PCR with a single enrichment step in APW as the best method for detecting *V. parahaemolyticus*. Improved results were obtained when this protocol was used in combination with the MPN technique. Conversely, a two-step enrichment protocol with sequential use of APW broth and SPB is highly recommended for the detection and enumeration of *V. parahaemolyticus* by plate culture, with CHROMagar Vibrio as the most appropriate selective agar for the isolation of *V. parahaemolyticus* in samples from the marine environment.

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