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Development of a PCR assay targeting the *rpoA* gene for the screening of *Vibrio* genus.

Running head: PCR detection of *Vibrio* genus.

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Abstract

A polymerase chain reaction (PCR) method based on the RNA polymerase alpha subunit (*rpoA*) gene was developed for the detection of *Vibrio* genus.

The specific primers were designed aligning the *rpo*A gene sequences available in GenBank of all *Vibrio* species.

The specificity of primers was tested against 35 *Vibrio* species. In addition 12 species phylogenetically related to *Vibrio* genus were used as negative control.

Moreover, in order to eliminate any false negative results, bacterium-specific primers, targeting the 16S rRNA gene, were introduced in the test as a noncompetitive Internal Amplification Control (IAC).

The *rpoA* primers correctly amplified all the *Vibrio* species considered. No cross reaction was observed when tested against closely related species.

To estimate the applicability of this method, 336 *Vibrio* wild type strains isolated from Italian aquaculture products and from imported seafoods were tested.

The sensitivity, tested using serial dilutions of different pure cultures of certified strains, resulted of 10^3 CFU/ml.

The assay proved to be specific, rapid and reliable. It can be proposed as a routine screening technique for the confirmation of *Vibrio* genus in isolated colonies.

Keywords: *rpoA*; *Vibrio* spp.; PCR.

1. Introduction

Various Gram-negative heterotropic bacteria may be isolated in seafood, such as *Aeromonas*, *Pseudomonas, Alcaligenes, Moraxella, Acinetobacter, Flavobacterium, Cytophaga, Chromobacterium, Photobacterium, Lucibacterium* and *Vibrio* (Cavallo et al. 2009). *Vibrio* species have been extensively studied for their implication in related public health hazards.

Vibrio genus comprises 74 species including 10 species pathogenic to humans: *V. cholerae, V. mimicus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, V. cincinnatiensis, V. vulnificus, V. furnissii, V. metschnikovii* and *V. harveyi* (*carchariae*) (Tarr et al. 2007; Noguerola and Blanch 2008). *V. cholerae, V. parahaemolyticus* and *V. vulnificus* can cause gastroenteritis, septicaemia and wound infections mostly through seafood consumption and handling; they are therefore considered significant zoonotic agents (Thompson et al. 2004; Ferrini et al. 2008).

Other *Vibrio* species are considered opportunistic invaders or weak pathogens of stressed fish, thus causing financial losses when infection appears in fish breedings (Ghittino et al. 2003). Among these, *V. anguillarum* is the most common bacterium affecting marine fish (Crosa et al. 2006; Hong et al. 2007). *V. ordalii* has been recognized as one of the major causes of vibriosis in wild and cultured marine salmonids in Japan and in the Pacific Northwest of the United States (Crosa et al. 2006).

Accurate phenotypic identification of *Vibrio* species is problematic, largely because of the great variability in their biochemical characteristics (Thompson et al. 2004; Tarr et al. 2007). A common example of misidentification involves *Aeromonas caviae*, often identified as *V. fluvialis* (Abbott et al. 2008). In addition, *Aeromonas hydrophila* and *Plesiomonas shigelloides* could also be mistaken for *Vibrio* when a complete battery of screening tests is not performed (Kwok et al. 2002).

For these reasons, more specific, rapid and sensitive molecular methods for *Vibrio* species identification are needed.

Virulence genes specific to some *Vibrio* species have been studied and used for identification of vibrios. For example, thermostable direct hemolysin gene (*tdh*) and thermostable direct hemolysin-related gene (*trh*) are well documented virulence markers of *V. parahaemolyticus*, while vulnificus hemolysin gene (*vvh*) is known as a marker for *V. vulnificus* (Bej et al. 1999; Blackstone et al. 2003;

Lee et al. 2003; Panicker et al. 2004). In addition, cholera toxin enzymatic subunit A (*ctxA*) and toxin-coregulated pilus (*tcpA*) genes have been used to detect toxigenic *V. cholerae* (Singh et al. 2002; Blackstone et al. 2007; Fedio et al. 2007).

On the other hand, many strains that do not contain known virulence markers have been recognized as causative agents of a large number of diarrhoeal cases (Singh et al., 2002). For instance, the majority of *V. cholerae* O1 or O139 pathogenic strains do not present virulence markers, thus making their identification difficult (Nandi et al. 2000). Likewise, a small portion of strains from clinical stool samples carried no reported virulence genes, resulting in their possible misidentification (Kim et al. 1999).

To address these difficulties, in the last few years new PCR methods have been explored. These methods target housekeeping genes, like peptidoglycan hydrolase N-acetylmuramoyl-L-alanine amidase (*amiB)*, *dnaJ*, gyrase B (*gyrB*), the collagenase or RNA polymerase subunits (*rpoA* and *rpoB*) genes (Di Pinto et al. 2005; Thompson et al. 2005; Kumar et al. 2006; Hong et al. 2007; Nhung et al. 2007; Tarr et al. 2007). Such approaches can reliably identify *Vibrio* strains, irrespective of their phenotypes, serotypes, and virulence status.

The aim of this study was the development of a rapid, reliable and specific diagnostic method based on PCR, targeting the *rpoA* gene, for the identification of *Vibrio* genus on suspected isolated colonies.

2.Materials and Methods

2.1 Samples

Certified strains belonging to 35 *Vibrio* species were used to develop the PCR assay (Table 1).

Furthermore, with reference to the *rpoA* phylogenetic tree obtained by Thompson et al. (2005), 12 species phylogenetically related to *Vibrio* genus, has been used (Table 1).

In order to evaluate the possible applicability of the proposed assay, 336 *Vibrio* wild type strains from in house strain collection were tested. In particular these strains were isolated from Italian aquaculture products and imported seafood in a time comprised between 2005 and 2007 (Table 2). For the isolation, 25 g of homogenized samples of fish or shellfish were diluted in 225 ml of

Alcaline Peptone Water Saline (APWS) and incubated at 31°C for 7-8 h. The broth (0.1 ml) was plated onto Thiosulphate Citrate Bile Salt Sucrose selective medium (TCBS, Oxoid, Cambridge, UK). Strains were further investigated using the following tests: Gram stain, API20NE (Biomerieux, Marcy l'Etoile, France), culture on triple sugar iron agar (TSI, Oxoid, Cambridge, UK), mobility and oxidase test. In a second time, to confirm species identification, wild type strains were subjected to the available species-specific PCR (Brauns et al. 1991; Kim et al. 1999; Nandi et al. 2000; Di Pinto et al. 2005; Chakraborty et al. 2006; Nhung et al. 2007) or to sequencing analysis with the MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems, Foster City, California, USA). Sequenced fragments were resolved by capillary electrophoresis using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The nucleotide sequences were submitted to BLASTN sequence similarity search at the National Centre of Biotechnology Information (NCBI) database (Altschul et al. 1990).

2.2 DNA extraction

The halophilic strains were grown aerobically overnight at 31°C in Marine Broth (Oxoid, Cambridge, UK), while the other strains were grown in Tryptic Soy Broth (TSB, Acumedia, Michigan, USA). DNA was extracted using the following protocol: 1 ml of broth culture was centrifuged at 12000 rpm for 5 min; the pellet was resuspended in 1 ml of sterile water, boiled for 5 min and centrifuged again. The supernatant was stored at -20°C until use.

DNA was quantified by means of a spectrophotometer (BioPhotometer 6131, Eppendorf AG, Hamburg, Germany).

2.3 Primers

The *rpo*A gene sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) of all *Vibrio* species were aligned in order to design *Vibrio* genus specific primers using ClustalX software (Higgins et al. 1992).

Furthermore, to evaluate possible cross-reactions, the sequences of some non *Vibrio* species (*Aeromonas* spp., *Plesiomonas* spp., *Photobacterium phosphoreum, Photobacterium damselae*) were also aligned.

Primers were designed as follows: 5'-AAATCAGGCTCGGGCCCT-3' (sense) and 5'-GCAATTTT(A/G)TC(A/G/T)AC(C/T)GG-3'(antisense), corresponding, respectively, to positions 294 to 311 and 519 to 535 of *V. parahaemolyticus* (GenBank accession number no. AJ842676).

Primers were designed in regions highly homologous and well conserved among all *Vibrio* species: they showed from 0 up to 2 mismatches with the Primers Binding Sites (PBS) of all *Vibrio* (Table 3).

Moreover, the bacterium-specific 16S rDNA primers (P63f and P518r) previously described by Dewettinck et al. (2001) were used as a noncompetitive Internal Amplification Control (IAC).

Primers were synthesized by Operon (Cologne, Germany).

2.4. Specificity

In a preliminary phase of this investigation, primers specificity was assessed by analysing DNA extracted from all reference strains by simplex *rpoA*-PCR. The PCR was carried out in a final volume of 50 µl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 Unit of Recombinant TAQ DNA Polymerase (Invitrogen, Paisley, UK), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Invitrogen, Paisley, UK), 2 mM di $MgCl_2$, 25 pmol of *rpoA* primer and 50 ng of DNA template.

The reaction was optimized for the target as previously described and subsequently the noncompetitive IAC was introduced, as indicated by Hoorfar et al.(2004).

The specificity of duplex PCR was also tested on DNA extracted from all reference strains in order to evaluate potentially undesirable pairings of primers. The duplex PCR reactions were performed in a 50 µl volume consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 Unit of Recombinant TAQ DNA Polymerase (Invitrogen, Paisley, UK), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Invitrogen, Paisley, UK), 2 mM di $MgCl_2$, 25 pmol of each primer and 50 ng of DNA template.

Thermocycling conditions were, both for simplex and duplex PCR, the following: initial denaturation at 94°C for 3 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min

annealing at 55°C and 1 min extension at 72°C. The final extension was carried out at 72°C for 5 min.

Amplification was performed in a GeneAmp PCR System 2720 thermal cycler (Applied Biosystems, Foster City, California, USA).

Amplimers were resolved by electrophoresis on a 2 % agarose gel (Invitrogen, Paisley, UK), run in Tris Acetate EDTA buffer for 70 min at 110V and stained with ethidium bromide (0.4 ng/ml) for 20 min.

Confirmatory sequencing of the amplified *rpoA* fragments was carried out for all reference strains. Amplified products were purified by means of Exo-Sap treatment according to the manufacturer's recommendations (USB Europe GmbH, Staufen, Germany). Forward and reverse sequencing reactions were performed using *rpoA* primers and ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, California, USA). Sequenced fragments were purified by DyeEX (Qiagen, Hilden, Germany) and resolved by capillary electrophoresis using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The nucleotide sequences were analyzed using the BLASTN sequence similarity search at the NCBI database (Altschul et al. 1990).

2.5 Sensitivity

The sensitivity was evaluated using pure cultures of *V. parahaemolyticus* ATCC 17802, *V. gallicus* CIP 107863 and *V. xuii* CIP 108271. These strains showed 0, 1, 2 mismatches between primers and PBS respectively (Table 3). Each bacterial strain was grown in 10 ml of Marine Broth (Oxoid, Cambridge, UK). After incubation at 31°C for 18 hours, bacteria were pelleted by centrifugation, washed three times and resuspended in sterile water to a final concentration of 10^8 CFU/ml.

Subsequently, the cultures were 10-fold serially diluted in sterile water up to 10 CFU/ml. DNA was extracted from the serial dilutions following the procedure described above. Five micro-litres of extracted DNA were used as a template in PCR.

3. Results

When the assay was performed on all *Vibrio* reference samples, the *rpoA* primers generated a 242 bp specific fragment and the bacterium-specific primers amplified a 456 bp amplicon (Fig.1A). Negative samples produced only the 456 bp fragment (Fig.1B).

The specificity of the amplified *rpoA* fragments was further confirmed when nucleotide sequences of all reference strains were submitted to BLASTN sequence similarity search (Altschul et al. 1990).

The validity of the method was demonstrated by confirming the identification at genus level of all 336 *Vibrio* wild type strains.

Finally, when the sensitivity test was carried out, the detection limit was 10^3 CFU/ml in all cases (Fig. 2).

4. Discussion

The availability of methods able to identify the *Vibrio* genus correctly is crucial to provide a better understanding of the ecology and the patterns of distribution of this microorganism in fish and shellfish products.

For this reason, in the past few years, several biomolecular techniques, able to identify these bacteria were proposed.

Some authors developed a PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) to distinguish the genus *Photobacterium* from *Vibrio* spp. (Urakawa et al. 1998). However the use of PCR-RFLP presents several drawbacks due to possible restriction site mutations or to a secondary conformation of DNA which could impair the access to the target site by the enzyme.

Others authors clearly differentiated *Vibrio* spp. from *Aeromonas* spp. by means of a sequence-based approach (Tarr et al. 2007). This technique, even though accurate, may not be suitable for routine analysis as it is expensive and requires experienced analyst.

The aim of the current study was to develop a biomolecular test able to rapidly identify *Vibrio* genus on suspected isolated colonies, without the need for further biochemical tests. So, a PCR test amplifying a *rpoA* gene fragment specific for vibrios was optimized.

The *rpoA* gene presents several advantages: i) it is widely distributed among genomes, ii) it belongs to the bacterial core genome, iii) it is a single copy gene, iiii) although it is highly conserved among the *Vibrio* genus, it shows a good variability against related genera such as *Grimontia*, *Listonella, Photobacteriaceae* or *Aeromonadaceae* and iiiii) it has been widely sequenced as it is used for phylogenetic and species identification studies (Thompson et al. 2005).

Based on the alignment of *rpoA* sequences of all *Vibrio* species, we designed primers in regions highly homologous and conserved among all *Vibrio* species and we selected 35 *Vibrio* species representative of all mismatch combinations between primers and PBS (Table 3).

The amplification of all these *Vibrio* species and in particular those with 2 mismatches (*V. gazogenes*, *V. pectenicida V. splendidus, V. xuii* and *V. rarus*), supported the choice of these primers.

Furthermore, the presence of several mismatches between primers and PBS in closely related species such as *Grimontia, Enterovibrio, Photobacterium* and *Aeromonas* avoided cross-reactions.

Moreover, *rpoA* primers correctly amplified DNA from all *Vibrio* wild type strains, thus confirming the robustness of primer design and the applicability of PCR to isolates retrieved during routine analyses.

To evaluate the possibility of proposing the application of this PCR as a screening technique, we also introduced a noncompetitive IAC in the reaction. An IAC is a nontarget DNA sequence present in the same sample reaction tube which is coamplified simultaneously with the target sequence. The inclusion of an IAC in an assay avoids the possibility of false negative results, due to the inhibition of the polymerase enzyme or to the malfunction of thermal-cycler. For this reason, several authors (Hoorfar et al. 2004; Rip and Gouws 2009) supported the importance of an IAC in PCR based methods to be used in routine analysis.

Finally, the sensitivity of the method seems to be not influenced by mismatches between primers and PBS. In fact, testing strains with 0, 1 and 2 mismatches the same level of sensitivity was highlighted.

However, given the values of sensitivity observed (10^3 CFU/ml), it would not be advisable to use PCR on DNA directly extracted from foods. However, this is not a problem, because *Vibrio*

detection always requires enrichment and consequent isolation of the bacterium by traditional techniques. In fact, since *Vibrio* species usually represent only a small fraction of the whole bacteria population naturally present in shellfish and fishery product, the application of the assay on isolated colonies would increase the chances of detecting *Vibrio* species.

In conclusion, the proposed PCR allows a rapid and accurate identification of *Vibrio* isolated colonies and can differentiate this genus from others aquatic microrganisms that could grow on the same media. Moreover, the developed test may represent a preliminary step prior to specific PCR methods for the screening of virulence factors as well as for the detection of pathogenic *Vibrio* species.

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Figure and table captions

Fig.1A. Evaluation of the specificity of the assay using *Vibrio* species DNA as template. Lane 1, *Vibrio cholerae*; lane 2, *Vibrio parahaemolitycus*; lane 3, *Vibrio vulnificus*; lane 4, *Vibrio mimicus*; lane 5; *Vibrio fluvialis*; lane 6, *Vibrio harveyi*; lane 7, *Vibrio alginolyticus*; M, 100-bp ladder.

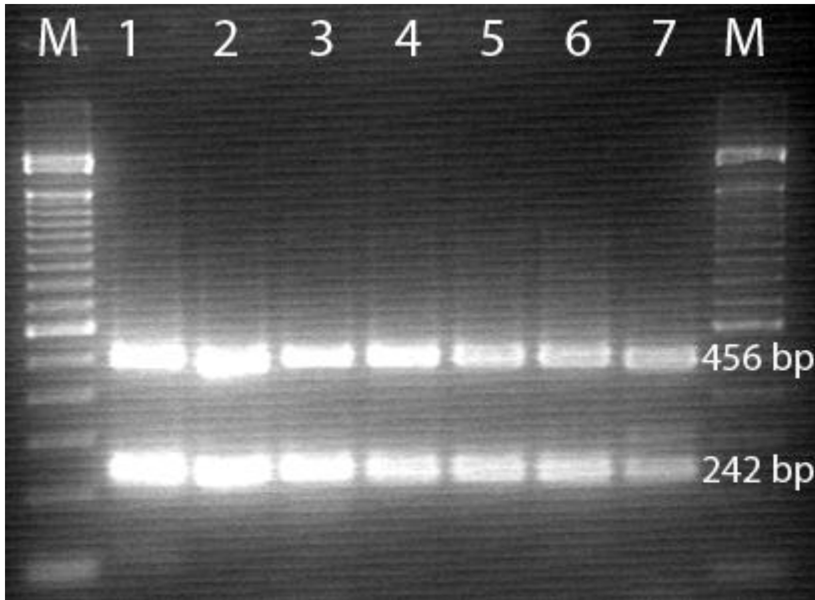

Fig.1B. Evaluation of the specificity of the assay using as template DNA of species phylogenetically related to *Vibrio* genus. Lane 1, *Aeromonas hydrophila*; lane 2, *Aeromonas caviae*; lane 3, *Aeromonas veronii*; lane 4, *Grimontia hollisae*; lane 5, *Aliivibrio fischeri*; lane 6, *Photobacterium phosphoreum*; lane 7, *Photobacterium damselae*; lane 8, *Plesimonas shigelloides*; lane 9, *Alteromonas macleodii*; lane 10, *V. cholerae*; W, control reagent; M, 100-bp ladder.

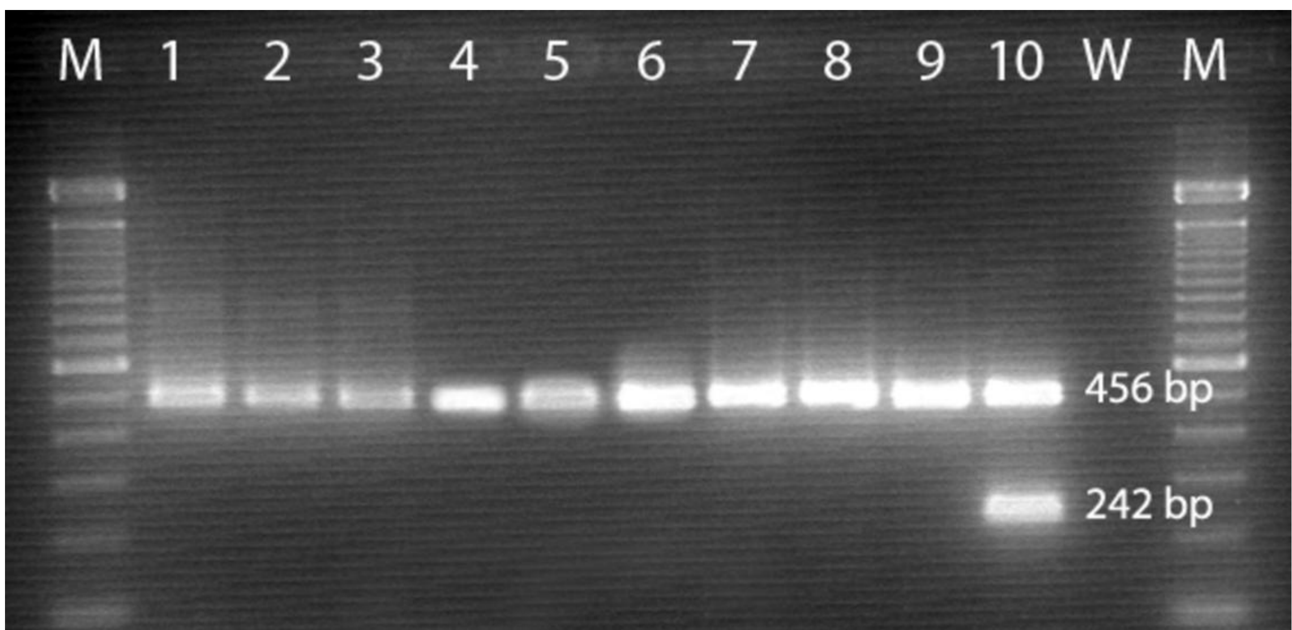

Fig.2. Evaluation of the sensitivity of the assay. Lane 1, 10^8 CFU/ml; lane 2, 10^7 CFU/ml; lane 3, 10^6 CFU/ml; lane 4, 10^5 CFU/ml; lane 5, 10^4 CFU/ml; lane 6, 10^3 CFU/ml; lane 7, 10^2 CFU/ml; lane 8, 10 CFU/ml; M, 100-bp ladder.

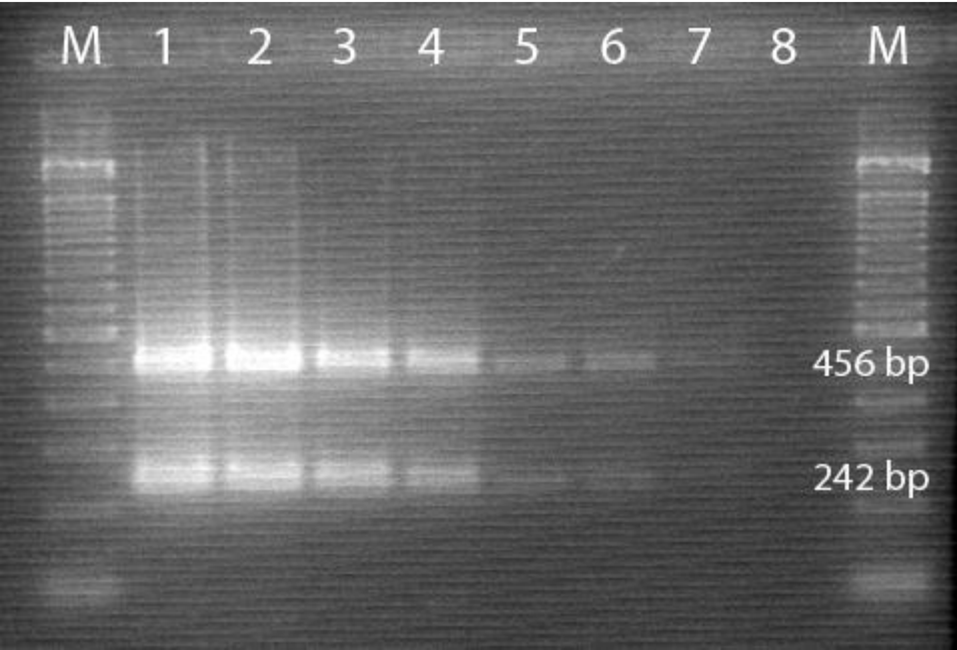

Table 1
Certified bacterial strains used to develop the assay.

Species	Source	Strain
Vibrio aestuarianus	CCUG [a]	28583T
Vibrio alginolyticus	ATCC [b]	17749
Vibrio alginolyticus	ATCC	19108
Vibrio alginolyticus	ATCC	33787
Vibrio anguillarum	CCUG	13446
Vibrio campbellii	ATCC	25920
Vibrio campbellii	BCCM/LMG [c]	11216
Vibrio carchariae (V. harveyi)	ATCC	35084
Vibrio cholerae	CCUG	33379
Vibrio cholerae	CCUG	45388
Vibrio cholerae	ATCC	9454
Vibrio cholerae	NCTC [d]	8042
Vibrio cholerae	ATCC	14035
Vibrio cincinnatiensis	BCCM/LMG	7891
Vibrio cincinnatiensis	ATCC	33912
Vibrio diabolicus	BCCM/LMG	23862
Vibrio diazotrophicus	ATCC	33466
Vibrio fluvialis	ATCC	33809
Vibrio furnissi	ATCC	33813
Vibrio furnissi	ATCC	35016
Vibrio gallicus	CIP	107863
Vibrio gazogenes	BCCM/LMG	19540
Vibrio harveyi	ATCC	14126
Vibrio ichthyoenteri	BCCM/LMG	19664
Vibrio mediterranei	CIP	103203
Vibrio metschnikovii	CCUG	30405
Vibrio metschnikovii	ATCC	7708
Vibrio mimicus	CCUG	48106
Vibrio mimicus	ATCC	33654
Vibrio mimicus	ATCC	33653
Vibrio mytili	ATCC	51288
Vibrio natriegens	BCCM/LMG	10935
Vibrio navarriensis	BCCM/LMG	15976
Vibrio nereis	BCCM/LMG	3895
Vibrio ordalii	CCUG	38425T
Vibrio pacinii	CIP	108197
Vibrio parahaemolyticus	ATCC	17802
Vibrio parahaemolyticus	CCUG	43364
Vibrio parahaemolyticus	CCUG	43363
Vibrio parahaemolyticus	ATCC	43996
Vibrio parahaemolyticus	ATCC	27519
Vibrio pectenicida	BCCM/LMG	19642
Vibrio proteolyticus	ATCC	15338
Vibrio rarus	BCCM/LMG	23674
Vibrio rotiferianus	CIP [e]	107930
Vibrio rumoiensis	DSMZ [f]	19141
Vibrio scophthalmi	BCCM/LMG	19158
Vibrio splendidus	ATCC	33125
Vibrio tubiashii	CCUG	38428
Vibrio tapetis	CIP	104856
Vibrio vulnificus	CCUG	13448T
Vibrio vulnificus	CCUG	47319
Vibrio vulnificus	ATCC	33149
Vibrio vulnificus	NCTC	11067
Vibrio xuii	CIP	108271
Aeromonas hydrophila	CCUG	44811
Aeromonas hydrophila	CCUG	14551T
Aeromonas hydrophila	CCUG	25940
Aeromonas hydrophila	CIP	103561
Aeromonas hydrophila	CIP	103697
Aeromonas hydrophila	CIP	57.50
Aeromonas caviae	CIP	63.24
Aeromonas caviae	CIP	102629
Aeromonas caviae	CIP	74.32
Aeromonas caviae	CCUG	25939T
Aeromonas caviae	CCUG	21694
Aeromonas salmonicida	CIP	103209T
Aeromonas sobria	CIP	74.33
Aeromonas veronii	CCUG	27821
Aeromonas veronii	CCUG	30362
Alteromonas macleodii	CCUG	16128
Photobacterium phosphoreum	BCCM/LMG	4233
Photobacterium damselae	BCCM/LMG	7892
Photobacterium damselae	ATCC	33536
Plesimonas shigelloides	CCUG	10616
Pseudomonas aeruginosa	CCUG	38935
Aliivibrio fischeri	BCCM/LMG	4414

Grimontia hollisae	CCUG	13625
Grimontia hollisae	ATCC	35912

[a] CCUG, Culture Collection University of Göteborg.
[b] ATCC, American Type Culture Collection.
[c] BCCM/LMG, Belgian Co-ordinated Collections of Micro-Organism.
[d] NCTC, National collection of Type Cultures.
[e] CIP, Collection de l'Institut Pasteur.
[f] DSMZ, German Collection of Microorganisms and Cell Cultures

Table 2

Vibrio wild type strains used to evaluate the applicability of the assay.

	Number of wild type strains	**Species**
Human pathogens	114	*V. alginolyticus*
	70	*V. parahaemolyticus*
	55	*V. cholerae*
	38	*V. harveyi*
	3	*V. fluvialis*
	2	*V. vulnificus*
	1	*V. mimicus*
Environmental strains and fish pathogens	18	*V. diabolicus*
	11	*V. ordalii*
	11	*V. anguillarum*
	6	*V. diazotrophicus*
	4	*V. rotiferianus*
	2	*V. mytili*
	1	*V. aestuarianus*

Table 3 Primers design based on alignment of sequences of all *Vibrio* species. Strains are arranged according to the number of mismatches between Primer Binding Sites (PBS) and primers.

	GenBank Acc. N°	PBS sense	PBS antisense	N° mismacthes
Primers		A A A T C A G G C T C G G G C C C T	G G Y C A D C T R T T T T A A C G	-
V. aestuarianus	AM884022	. . . .	. . . .	0
V. alginolyticus	AJ842559	. . . .	. . . .	0
V. anguillarum	AJ842561	. . . .	. . . .	0
V. cholerae	AJ842581	. . . .	. . . .	0
V. diabolicus	AJ842594	. . . .	. . . .	0
V. diazotrophicus	AJ842597	. . . .	. . . .	0
V. fluvialis	AJ842606	. . . .	. . . .	0
V. furnissii	AJ842614	. . . .	. . . .	0
V. harveyi	AJ842628	. . . .	. . . .	0
V. metschnikovii	AJ842652	. . . .	. . . .	0
V. mimicus	AJ842653	. . . .	. . . .	0
V. mytili	AJ842657	. . . .	. . . .	0
V. natriegens	AJ842658	. . . .	. . . .	0
V. ordalii	AJ842669	. . . .	. . . .	0
V. parahaemolyticus	AJ842676	. . . .	. . . .	0
V. proteolyticus	AJ842686	. . . .	. . . .	0
V. hispanicus	AJ842632	. . . .	. . . .	0
V. tapetis	AJ842730	. . G	. . . .	1
V. breoganii	EU541576	. . . . A	. . . .	1
V. comitans	EU541577	. . . . A	. . . .	1
V. ezurae	AJ842601	. . . . A	. . . .	1
V. gallicus	EU541581	. . . . A	. . . .	1
V. halioticoli	AJ842619	. . . . A	. . . .	1
V. inusitatus	EU541579	. . . . A	. . . .	1
V. mediterranei	AJ842646	. . . . A	. . . .	1
V. neonatus	AJ842662	. . . . A	. . . .	1
V. nigripulchritudo	AJ842667	. . . . A	. . . .	1
V. shilonii	AJ842695	. . . . A	. . . .	1
V. superstes	AJ842727	. . . . A	. . . .	1
V. campbellii	AJ842569	. . . .	. . T	1
V. cincinnatiensis	AJ842582	. . . .	. . T	1
V. navarrensis	AJ842659	. . . .	. . T	1
V. penaeicida	AJ842683	. . . .	. . T	1
V. porteresiae	EU072028	. . . .	. . T	1
V. rotiferianus	AJ842688	. . . .	. . T	1
V. vulnificus	AJ842737	. . . .	. . T	1
V. pacinii	AJ842674	. . . .	. . . . . G	1
V. aerogenes	AJ842553	. . . .	. . . . G	1
V. brasiliensis	AJ842563	. . . .	. . . . G	1
V. coralliilyticus	AJ842588	. . . .	. . . . G	1
V. hepatarius	AJ842631	. . . .	. . . . G	1
V. ichthyoenteri	AJ842633	. . . .	. . . . G	1
V. neptunius	AJ842664	. . . .	. . . . G	1
V. nereis	AJ842666	. . . .	. . . . G	1
V. orientalis	AJ842672	. . . .	. . . . G	1
V. scophthalmi	AJ842693	. . . .	. . . . G	1
V. tubiashii	AJ842735	. . . .	. . . . G	1
V. rumoiensis	AJ842691	. . . .	. . . . C . .	1
V. chagasii	AJ842572	. . . . A	. . . . C . .	2
V. crassostreae	EU541574	. . . . A	. . . . C . .	2
V. cyclitrophicus	AJ842593	. . . . A	. . . . C . .	2
V. fortis	AJ842609	. . . . A	. . . . C . .	2
V. gallaecicus	EU931118	. . . . A	. . . . C . .	2
V. gigantis	EU541573	. . . . A	. . . . C . .	2
V. kanaloae	AJ842637	. . . . A	. . . . C . .	2
V. lentus	AJ842639	. . . . A	. . . . C . .	2
V. pomeroyi	AJ842684	. . . . A	. . . . C . .	2
V. splendidus	AJ842725	. . . . A	. . . . C . .	2
V. tasmaniensis	AJ842731	. . . . A	. . . . C . .	2
V. rarus	EU541578	. . . . A	. . T	2
V. xuii	AJ842742	. . . . A	. . . . G	2
V. gazogenes	AJ842616	. . . .	. . . . . G . . . . C . .	2
V. rhizosphaerae	EF523233	. . . .	. . . . . G . . . . C . .	2
V. ruber	EF523235	. . . .	. . . . . G . . . . C . .	2
V. pectenicida	AJ842680	. . . .	. . . . G . . A . .	2