Multiplex primer-extension assay for identification of six pathogenic vibrios

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Abstract

A multiplex Primer-Extension Reaction (PER) assay, was specifically designed for the identification, of the major human pathogenic *Vibrio* species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus* and *V. fluvialis*) in fishery products. The assay, directed towards the *rpoA* gene, was tested on a total of 287 samples representing six *Vibrio* species and ten non-*Vibrio* species. The primers used in the preliminary PCR, designed in well conserved regions upstream and downstream of the diagnosis sites, successfully amplified a 284 bp fragment. The diagnosis sites were simultaneously interrogated using a multiplex PER and the results were confirmed by fragment sequencing. The proposed test provides an appropriate tool to monitor the presence of these human pathogenic species in seafood samples and to evaluate the potential hazard for consumers.

Keywords: *Vibrio*; *rpoA*; Species identification; Multiplex Primer-Extension Reaction.

Introduction

In the last few years, a considerable rise of *Vibrio* diseases in many developed countries has been recorded. Possible causes are the expansion of the international trade of fishery products and/or the increase in their consumption after an insufficient purification procedure during processing (Tantillo et al., 2004). The Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) estimated a 126% increase in the incidence of *Vibrio* associated infection between 1996 and 2002, in spite of the repeated actions the Authorities undertake in order to make people more aware of the hazard connected with the consumption of raw seafood (CDC, 2003).

The *Vibrio* genus is characterized by a large number of species; some of these are human pathogens causing gastrointestinal and wound infections through the ingestion or manipulation of contaminated fishes and shellfish (Thompson et al., 2004). *V. cholerae* is the etiological agent of cholera, which is spread by contaminated food, water or direct faecal contact with food handlers. Shellfish are mainly responsible for its transmission (Tarr et al., 2007). The presence of *V. vulnificus* has been well documented in water and molluscs and infections have been associated with consumption of raw oysters and contact with shellfish (Croci et al., 2001). *V. parahaemolyticus* has been recognized as an important cause of foodborne disease due to the consumption of raw or undercooked seafood in Asia and North America. In Europe, disease caused by *V. parahaemolyticus* have rarely been reported and little information regarding the prevalence in the environment is available (Croci et al., 2001; Robert-Pillot et al., 2004). Besides these well known species, other vibrios (*V. fluvialis*, *V. alginolyticus*, *V. mimicus*) responsible for sporadic infections, have been sometimes isolated in many countries (Oliver and Kaper, 2001). In particular, *V. fluvialis* is usually associated with the consumption of raw or improperly cooked seafood (Kothary et al., 2003), while *V. alginolyticus* and *V. mimicus* are infrequently reported as cause of foodborne disease (Wei et al., 2008).

Since the epidemiological studies of pathogenic species depend on their unmistakable identification, accurate methods are needed. A great number of traditional microbiological techniques have been developed; they are based on the culture on selective media. However they require confirmatory assays based on biochemical, serological and biomolecular test. Recent advances in molecular biology have introduced new techniques for the detection of microbial species such as the amplification of species-specific fragments (Di Pinto et al., 2005; Nhung et al., 2007; Tarr et al., 2007) or the analysis of the diagnosis sites (Reen and Boyd, 2005; Saha et al., 2006). When closely related species are considered, the analysis of Single Nucleotide Polymorphism (SNP) seems to be the best approach. In these cases biomolecular assays such as the use of restriction enzymes (Saha et al., 2006), TaqMan Minor Groove Binding (MGB) probes (Easterday et al., 2005; U'Ren et al., 2005) and sequencing primers associated with fluorescently labelled dideoxy-nucleotide ([F]ddNTPs) (Scott et al., 2007) have been successfully applied. In particular, this latter technique is based on the simultaneous analysis of several diagnosis sites by means of multiplex Primer-Extension Reaction (PER) in conjunction with a genetic analyser. The PER reaction is based on the addition of a [F]ddNTPs to the 3′ end of an unlabeled oligonucleotide (sequencing primer) in the absence of dNTPs in the reaction. Each sequencing primer is designed immediately adjacent to the diagnosis sites and their length is modified by the addition of non-homologous tails at the 5′ end. The extended sequencing primers used to interrogate the diagnosis sites differ by size and colour which is specific to diagnosis site (Quintans et al., 2004).

The aim of the study was the development of the above described assay for differentiation of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, *V. alginolyticus* and *V. mimicus*. This test could represent a valid tool for the study of the risk factors connected with the main pathogenic *Vibrio* in seafood.

2. Materials and methods

2.1. Samples

All reference species of *Vibrio* (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, *V. alginolyticus* and *V. mimicus*) used to develop the assay are listed in Table 1. Bacterial strains belonging to phylogenetically related genera were also tested to verify possible cross-reactions (Table 1). Finally, in order to evaluate the possible applicability of the proposed assay, 245 wild isolates (WI) (55 *V. cholerae*, 70 *V. parahaemolyticus*, 2 *V. vulnificus*, 3 *V. fluvialis*, 114 *V. alginolyticus* and 1 *V. mimicus*) obtained from Italian aquaculture products and imported seafood were used. The samples, supplied by Istituto Zooprofilattico del Piemonte, Liguria e Valle d'Aosta, Istituto Superiore di Sanità, the Veterinary Medicine Faculties of Messina, Pisa and Sassari, were isolated in a time comprised between 2005 and 2007. For the isolation of WI, 25 g of homogenized sample were diluted in 225 mL of Alcaline Peptone Water Saline (APWS) and incubated at 31 °C for 7–8 h. The broth culture (0.1 mL) was plated onto Thiosulphate Citrate Bile Salt Sucrose selective medium (TCBS, Oxoid, Hampshire, England). Isolates were further investigated using the following tests: Gram stain, API20NE (Biomerieux, France), culture on Triple Sugar Iron agar (TSI, Oxoid, Hampshire, England), mobility and oxidase tests. Successively, the isolated colonies were identified by means of the species-specific PCR described by Nandi et al. (2000) for *V. cholerae*; by Kim et al. (1999) for *V. parahaemolyticus*; by Brauns et al. (1991) for *V. vulnificus*; by Chakraborty et al. (2006) for *V. fluvialis*; by Di Pinto et al. (2005) for *V. alginolyticus* and by Nhung et al. (2007) for *V. mimicus*.

2.2. DNA extraction

All the isolates were grown overnight at 31 °C in Marine Broth (Oxoid, Cambridge, UK) or Tryptone Soya Broth (TSB, Acumedia, Michigan, USA). DNA was extracted using the following protocol: 1 mL of broth culture was centrifuged at 12000 g for 5 min; the pellet was resuspended in 1 mL of Phosphate Buffered Saline (PBS), 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) and diluted at a concentration of 50 ng/µL.

2.3. Primers design

The *rpoA* sequences obtained from the GenBank database (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov), corresponding to *V. cholerae* (accession no. AJ842581), *V. parahaemolyticus* (accession no. AJ842677), *V. vulnificus* (accession no. AJ842737), *V. fluvialis* (accession no. AJ842606), *V. alginolyticus* (accession no. AJ842559) and *V. mimicus* (accession no. AJ842656), were aligned with the ClustalV program (Higgins et al., 1992) for the detection of polymorphic sites to be used as diagnosis positions (Table 2). All the sequences available for each species in GenBank (17, 3, 2, 3, 5 and 5 sequences for *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, *V. alginolyticus* and *V. mimicus*, respectively) were then examined to

confirm the absence of intraspecific variations of the diagnosis sites that have been selected. Primers for the preliminary PCR have been designed in well conserved regions upstream and downstream from the diagnosis sites (**Tables 2 and 3** [Table 2, Table 3]). Primers were synthesized by Operon (Cologne, Germany). Each sequencing primer (V1, V2, V3, V4, V5, V6) was designed upstream from the diagnosis sites and had varying lengths of poly(dT) non-homologous tails attached to the 5′ end (Tables 2 and 3). Sequencing primers were synthesized by SIGMA (St. Louis, Missouri, USA).

2.4. Preliminary PCR

PCR reactions were performed in 50 µL volumes consisting of 20 mM Tris–HCl (pH 8.4), 1 Unit of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, California, USA), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Invitrogen, Carlsbad, California, USA), 2 mM $MgCl_2$, 25 pmol of each primer and 50 ng of DNA template. Thermocycling conditions consisted of an initial denaturation at 94 °C for 10 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °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). Amplicons were resolved by electrophoresis on a 2.5% agarose gel (Invitrogen, Carlsbad, California, USA), run in tris acetate EDTA buffer for 70 min at 110V and stained with ethidium bromide (0.4 ng/mL) for 20 min.

The obtained PCR products act as templates for PER reaction after the enzymatic clean-up. To remove primers and unincorporated dNTPs, 2 µL of Exo-Sap (USB Europe GmbH, Staufen, Germany) were added to 5 µL of PCR products. Tubes were incubated at 37 °C for 1 h and 80 °C for 15 min.

2.5. PER

Tailed sequencing primers were tested individually to assess performance and validate migration size before the six primers were tested in multiplex reaction. Singleplex PER reactions were performed following the SNaPshot multiplex Kit protocol (Applied Biosystems, Foster City, California, USA) with minor modifications: 2.5 µL of SNaPshot Multiplex Ready Reaction Mix; 3 µL of purified preliminary PCR products diluted to obtain a range of 0.4–0.01 pmol; and 0.2 µM of sequencing primer in a total volume 10 µL.

Afterwards, a multiplex PER was performed using the following concentrations of sequencing primers: 0.3 µM of V1 and V2, 0.15 µM of V3 and V4, 0.0375 µM of V5 and V6. Primer concentrations varied depending on the efficiency of each primer and were selected after the preliminary assays. All reactions underwent 25 single base extension cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C during 30 s and were carried out in a GeneAmp PCR System 2720 thermal cycler (Applied Biosystems, Foster City, California, USA).

Subsequently, a post-extension treatment to remove the 5′-phosphoryl group of the [F]ddNTPs helps to prevent un-incorporated [F]ddNTPs co-migrating with the extended primers and producing a high background signal. For this purpose the final volume (10 µL) was treated with 1 unit of Calf Intestine Alkaline Phosphatase (CIAP) (Fermentas, Burlington, California, USA). The tubes were incubated at 37 °C for 1 h and at 75 °C for 15 min.

Finally, samples were prepared by adding 1 µL of the post-PER product to 24.6 µL of formamide (Applied Biosystems, Foster City, California, USA) and 0.4 µL of GeneScan 120 LIZ size standard (Applied Biosystems, Foster City, California, USA). Each sample was loaded on the ABI 310 Genetic Analyser (Applied Biosystems, Foster City, California, USA) and analysed using Pop4 polymer (Applied Biosystems, Foster City, California, USA), a 47-cm capillary column (Applied Biosystems, Foster City, California, USA), the ABI GeneScan E5 Run Module. Parameters for this analysis were as follows: injection time 12 s, run time 15 min. Electropherograms were analysed using the GeneScan 3.1.2 software (Applied Biosystems, Foster City, California, USA).

2.6. DNA sequencing

DNA sequencing was performed as a confirmatory test. Amplification products of the preliminary PCR of samples were sequenced. Sequencing was carried out directly on purified fragments with ABI 310 Genetic Analyser (Applied Biosystems, Foster City, California, USA), using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, California, USA). The nucleotide sequences were submitted to BLASTN (Altschul et al., 1990) sequence similarity search at the NCBI database and were aligned with the gene sequences available in GenBank database.

3. Results and discussion

The increase of *Vibrio* infections due to the consumption and the manipulation of contaminated fish and shellfish has made necessary the precise identification of *Vibrio* spp.

A correct monitoring of *Vibrio* species in seafood and in fish products could open new interesting perspectives for epidemiological studies on the principal risk factors associated with these microorganisms.

The aim of our study was to develop an assay for the specific identification of the six *Vibrio* species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, *V. alginolyticus* and *V. mimicus*) targeting the *rpoA* gene.

The *rpoA* gene has already been used in a multilocus sequencing strategy for phylogenetic studies and species identification (Thompson et al., 2005). This locus has several advantages: it belongs to the bacterial core genome, it is a single copy gene and presents no identical gene sequences in different species of vibrios, thus enabling a unambiguous identification of these organisms (Harris et al., 2003).

Analysis of the alignment of the six reference sequences obtained from GenBank showed that the bases in 287, 371, 388, 398, 466 and 467 positions of *rpoA* gene could differentiate *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, *V. alginolyticus* and *V. mimicus*, (Table 2). As the primers of the preliminary PCR have been designed in well conserved regions, amplification of other *Vibrio* species could occur. Therefore, the specificity of the selected diagnosis positions was further confirmed aligning *rpoA* gene sequences of all species of the genus. Moreover, the absence of intraspecific variation of the chosen sites was verified by the alignments of all the sequences available in GenBank for each species object of this study.

Preliminary PCR allowed the amplification of a 284 bp fragment in all reference *Vibrio* samples. No cross-reaction was detected when the primers were tested with other genera phylogenetically related to *Vibrio* genus. When DNA was challenged in singleplex PER, it gave rise to a peak of the expected colour which is specific to the [F]ddNTPs incorporated: green (adenine), black (cytosine), blue (guanine) and red (thymine).

The multiplex PER was optimized and the DNA extracted from six *Vibrio* species gave species-specific patterns (Fig. 1). To optimize the resolution of the profile in capillary electrophoresis, the length of the sequencing primers had to be increased of at least 6 nucleotides (dT). The sequencing primers ranged in size from 20 bp to 54 bp, although migration did not exactly correspond to primers size, in agreement with what observed in other works (Bottero et al., 2007; La Neve et al., 2007).

All of the reference strains and WI gave rise to the expected species-specific patterns thus confirming the applicability of the multiplex PER technique. Moreover, the absence of intraspecific variations in the numerous samples analysed (245 WI), confirmed the choice of the diagnosis site, further supported by sequencing results.

In conclusion, the multiplex PER assay is as precise and reliable as the sequencing test and it has the advantage of allowing an immediate interpretation of the result. In fact, the discrimination of the six species is possible by means of a single reading of the generated pattern. The likelihood of the simultaneous interrogation of different diagnostic sites and the high automation potential, make this assay a valid alternative to PCR-RFLP, especially when several species must be identified; in fact PCR-RFLP is difficult to automate due to the high number of necessary enzymes (Wolf et al., 1999). Recently, fluorescent methods such as Real Time PCR have been employed for the analysis of the diagnosis sites (Easterday et al., 2005; U'Ren et al., 2005). These methods have several advantages: they are rapid, allow the quantification of the results and eliminate the post-PCR manipulation. However they do not allow the simultaneous detection of all the species in the same tube.

The test proposed in this paper, applied for the specific detection of pathogenic *Vibrio*, represents the ideal solution for routine analysis in laboratories equipped with genetic analyser.

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Table 1: Bacterial strains used to develop the multiplex PER assay

Species	**Source**	**Strain**
Vibrio alginolyticus	ATCC [a]	17749
Vibrio alginolyticus	ATCC	19108
Vibrio alginolyticus	ATCC	33787
Vibrio cholerae	CCUG [b]	33379
Vibrio cholerae	CCUG	45388
Vibrio cholerae	ATCC	9454
Vibrio cholerae	NCTC [c]	8042
Vibrio cholerae	ATCC	14035
Vibrio fluvialis	ATCC	33809
Vibrio mimicus	CCUG	48106
Vibrio mimicus	ATCC	33654
Vibrio mimicus	ATCC	33653
Vibrio parahaemolyticus	ATCC	17802
Vibrio parahaemolyticus	CCUG	43364
Vibrio parahaemolyticus	CCUG	43363
Vibrio parahaemolyticus	ATCC	43996
Vibrio parahaemolyticus	ATCC	27519
Vibrio vulnificus	CCUG	13448T
Vibrio vulnificus	CCUG	47319
Vibrio vulnificus	ATCC	33149
Vibrio vulnificus	NCTC	11067
Aeromonas hydrophila	CCUG	44811
Aeromonas hydrophila	CCUG	14551T
Aeromonas hydrophila	CCUG	25940
Aeromonas hydrophila	CIP [d]	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 [e]	4233
Photobacterium damselae	BCCM/LMG	7892
Photobacterium damselae	ATCC	33536
Plesimonas shigelloides	CCUG	10616
Pseudomonas aeruginosa	CCUG	38935

[a] ATCC, American Type Culture Collection (Teddington, Middlesex, UK).

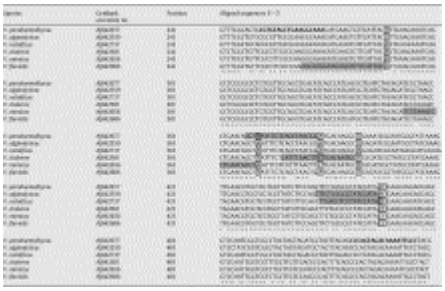
[b] CCUG,Culture Collection University of Göteborg (Göteborg, Sweden).

[c] NCTC, National Collection of Type Cultures (London, UK).

[d] CIP, Collection de l'Institut Pasteur (Paris, France).

[e] BCCM/LMG, Belgian Co-ordinated Collections of Micro-Organism (Gent, Belgium).

Table 2: Primers binding site for preliminary PCR primers and for PER primers



Primers binding site for preliminary PCR primers (in bold) and for PER primers (in grey). The bases in the box correspond to the sites of diagnosis.

Table 3: Primers for preliminary PCR and PER

	Primers	**Position (AJ842677)**	**Oligonucleotides**
Preliminary PCR	Sense	252	5′-CGCGTWGCCGAAGGCAAA-3′
	Antisense	536	5′-GGCAATTTTGTCTACTGGG-3′
PER	V1	368	5′-CGTTATTTGTCACYTAACKK-3′
	V2	378	5′-7(T)CACYTAACKKCTGACAAYGC-3′
	V3	448	5′-16(T)CTTCAGCCCGTATCCATA-3′
	V4	449	5′-22(T)TTCAGCCCGTATCCATAM-3′
	V5	264	5′-24(T)GGCAAAGATGAAGTGTTCATTAC-3′
	V6	352	5′-35(T)TCGYWAACCCTGAACACGT-3′

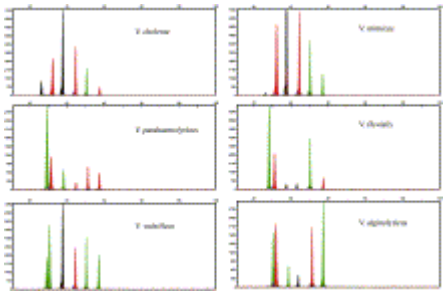


Fig. 1: Species specific patterns observed in reference strains.