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In-house validation of novel multiplex real-time PCR gene combination for the simultaneous detection of the main human pathogenic vibrios (*Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*)

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In recent years the incidence of vibriosis has greatly increased, raising the concern among consumers about the innocuity of certain food products. Previous studies demonstrated various advantages of molecular methods, including qPCR, for the screening of food-borne pathogens. The new method developed in the present study allows fast and reliable detection of the main human pathogenic *Vibrio* species (*Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*). Specificity of the combination of primers and probes was successfully tested against several bacterial species and strains (44 different strains). Evaluation of the qPCR efficiency reported a value of 94% with the simultaneous amplification of the internal amplification control. The evaluation of the quality of the method was based on six parameters: relative sensitivity, specificity, accuracy, positive and negative predictive values as well as Kappa index of concordance. Each of the values obtained were higher than 96%. Additionally a very low limit of detection was determined for the developed method (less than 10 cfu/25 g). All the parameters of the method analyzed were obtained from the analysis of a wide variety of foodstuffs, water samples and reference material from proficiency tests, and compared against the culture reference method.

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1. Introduction

The genus *Vibrio* comprises more than 70 species, that are ubiquitous in aquatic environments and inhabit marine animals. Thirteen different species have been associated with human pathogenesis, however, the majority of human *Vibrio* infections are associated with three species: *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* (Drake, DePaola, & Jaykus, 2007; Izumiya et al., 2011; Neogi et al., 2010; Thompson, Iida, & Swings, 2004).

V. cholerae is the causative agent of Asiatic or epidemic cholera. Two serogroups (O1 and O139) are responsible for cholera epidemics but others (non-O1/non-O139) have been associated with sporadic gastroenteritis (Blackwell & Oliver, 2008; Jones & Oliver, 2009; Neogi et al., 2010). The first report of *V. parahaemolyticus* infection was documented in 1950, and since then it has become the leading cause of seafood-derived food poisoning worldwide. Infection causes acute gastroenteritis, but it may also cause wound infection and septicemia (Broberg, Calder, & Orth, 2011; Hiyoshi, Kodama, Iida, & Honda, 2010; Su & Liu, 2007). Finally, *V. vulnificus* has been identified as the deadliest, in terms of case-fatality rate, food-borne pathogen in the US, accounting for 95% of all seafood related deaths. Its fatality rate among immunocompromised patients or individuals with underlying chronic disease can be as high as 50%. It may also cause gastroenteritis, necrotizing fasciitis, and wound infections (Gulig et al., 2010; Roig, Sanjuan, Llorens, & Amaro, 2010).

European regulation does not consider analysis of pathogenic vibrios in food, not even in its latest amendments ((EC), 2005, 2007, 2010). In contrast, the Canadian and American regulations specifically mention the main pathogenic *Vibrio* spp. (Canadian Food Inspection Agency; FDA, 2011).

At present several official methods exist for the screening of pathogenic vibrios in food, like those developed by the Internal Standardization Organization (ISO) or by the Food and Drug Administration (FDA) (Kaysner and Angelo DePaola, 2004, chap. 9; ISO 2007a, 2007b). Additionally, other newly developed methods,

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Table 1
Strains used for the evaluation of the specificity of primers and probes, and for the inoculation of food samples.

Bacteria	Strain	qPCR		
		ompW	*tlh*	*vvhA*
Vibrio parahaemolyticus	CECT 511[a], CECT 5271, CCUG 43362, CCUG 43363, CCUG 43364, CCUG 43365, CAIM 58	–	+	–
Vibrio cholerae	CECT 514[a] (O1), CCUG 47460 (O139), 1789[b] (algae), 8053c (pangasius)[b], 8053o (pangasius)[b]	+	–	–
Vibrio vulnificus	CAIM 611, CECT 529[a], CECT 4869, CECT 4608	–	–	+
Vibrio alginolyticus	CECT 586, CAIM 342, (unknown)[b]	–	–	–
Vibrio mimicus	CECT 4218, BCCM/LMG 7896	–	–	–
Aeromonas hydrophila	CECT 839	–	–	–
Pseudomonas putida	CECT 324	–	–	–
Pseudomonas aeruginosa	CECT 108	–	–	–
Pseudomonas fluorescens	CECT 378	–	–	–
Escherichia coli	CECT 516, CECT 434	–	–	–
Citrobacter freundii	CECT 401	–	–	–
Staphylococcus aureus	CECT 240, CECT 435	–	–	–
Salmonella enterica	CECT 4594	–	–	–
Salmonella spp.	311[b] (fishmeal), 312[b] (fishmeal), 313[b] (fishmeal), 314[b] (fishmeal), 315[b] (fishmeal)	–	–	–
Listeria monocytogenes	CECT 935, 810[b] (mussel)	–	–	–
Listeria innocua	CECT 910	–	–	–
Listeria seeligeri	CECT 917	–	–	–
Listeria ivanovii	CECT 913	–	–	–
Shigella sonnei	CECT 413	–	–	–
Shigella flexneri	CECT 4804	–	–	–
Enterococcus faecalis	CECT 481	–	–	–

[a] Reference strains for the evaluation of the method.
[b] Strain identified in our laboratory, if known, the source and/or serotype was indicated in parenthesis. CECT: Spanish Type Culture Collection, CCUG: Culture Collection University of Göteborg, CAIM: Collection of Aquatic Important Microorganisms, BCCM/LMG: Belgian Co-Orfinated Collections Of Micro-Organsims.

like those described by Williams, Froelich, and Oliver (2013) and Griffitt and Grimes (2013) (Williams et al., 2013), have been described. All these protocols are culture-based, and even though reliable, present several limitations like not being able to detect viable but not culturable bacteria (VBNC), are time-consuming, overgrowth of other bacteria may hide desired colonies or make the isolation step difficult, atypical biochemical profiles may not be correctly identified, among others (Chomvarin et al., 2007; Fedio et al., 2007; Lyon, 2001; Saravanan, Kumar, Karunasagar, & Karunasagar, 2007). All these limitations may be overcome by the application of molecular methods from which PCR/real-time PCR (qPCR) is the most popular (Bauer & Rorvik, 2007; Blanco-Abad, Ansede-Bermejo, Rodriguez-Castro, & Martinez-Urtaza, 2009; Malayil, Turner, Mote, Howe, & Lipp, 2011; Neogi et al., 2010; Sharma & Chaturvedi, 2006; Sheikh, Goodarzi, & Aslani, 2012). Furthermore, qPCR has been successfully used to detect and quantify food-borne pathogens, including pathogenic vibrios (Chapela et al., 2010; Gubala, 2006; Gubala & Proll, 2006; Kamio, Hara-Kudo, Miyasaka, Yahiro, & Konuma, 2008; Takahashi, Iwade, Konuma, & Hara-Kudo, 2005; Tyagi, Saravanan, Karunasagar, & Karunasagar, 2009). Even though a wide number of studies have been published targeting each individual bacterium, alone or combined with several virulence factors, multiplex qPCR methods for simultaneous detection at the species level of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are scarce. Furthermore, the sequence of primers and probes are not always freely available since some are included in commercial detection kits [(Tebbs, Brzoska, Furtado, & Petrauskene), BAX® System Real-Time PCR assay for Vibrio (DuPont Qualicon, Wilmington DE)].

The aim of the present study was to develop and evaluate an open straightforward multiplex qPCR method for simultaneous detection of the three most important human pathogenic vibrios using a novel combination of species-specific genes. The complete method consisted of direct sample enrichment in Alkaline Saline Peptone Water (ASPW), and a simple DNA extraction step prior to multiplex qPCR for the simultaneous detection of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. The method included as well an Internal Amplification Control to evaluate possible matrix-associated inhibition of the qPCR reaction.

2. Materials and methods

2.1. Bacterial strains and culture media

V. cholerae CECT 514, *V. parahemolyticus* CECT 511, and *V. vulnificus* CECT 529 were selected as reference strains for the evaluation of the method. These microorganisms were purchased by the Spanish Type Culture Collection (CECT). Bacteria were stored frozen at −20 °C until use. All other organisms used in this study, and their origin are summarized in Table 1.

Fresh cultures of all strains used in the present study were obtained by inoculating 10 mL tubes of Tryptic Soy Broth (TSB, BioMérieux S.A., France), and incubated at 37 °C overnight, except for *Bacillus subtilis* which was incubated at 31 °C.

2.2. Culture method for detection of pathogenic Vibrio spp.

Detection of *V. cholerae*, *V. parahemolyticus*, and *V. vulnificus* by traditional microbiology was carried out following the methods described in ISO/TS 21872-1: 2007 (method for detection of *V. cholerae* and *V. parahaemolyticus*) and ISO/TS 21872-2: 2007 (method for detection of potentially pathogenic vibrios other than *V. cholerae* and *V. parahaemolyticus*) (ISO, 2007a, 2007b). Briefly, both methods consisted of two enrichment steps in Alkaline Saline Peptone Water (ASPW, Biolife Italiana S.r.l., Italy), and plating on solid selective media. Thiosulphate Citrate Bile salt Sucrose (TCBS, OXOID, Hampshire, England) is used in both methods for isolation of these bacteria. A second isolation medium is recommended as well, either to be chosen by the laboratory (ISO, 2007a) or to be selected among a list of media (ISO, 2007b).

In the present study TCBS and CHROMagar™ Vibrio (CHROMagar Microbiology, Paris, France), were used for the isolation of *V. cholerae* and *V. parahaemolyticus*. Colistin Polymixin β-Cellobiose agar (CPC, Sigma–Aldrich, St. Louis, USA) was selected as the secondary isolation medium for *V. vulnificus*. The initial study also included HiCrome™ Vibrio (HC, Sigma–Aldrich, St. Louis, USA) as a possible secondary selective medium. A schematic presentation of the method is shown in Fig. 1.

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