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# Development of a colony hybridization method for the enumeration of total and potentially enteropathogenic *Vibrio parahaemolyticus* in shellfish

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ABSTRACT

*Vibrio parahaemolyticus* is a marine microorganism, recognized as cause of gastroenteritis outbreaks associated with seafood consumption.

In this study the development and the in-house validation of a colony hybridization method for the enumeration of total and potentially pathogenic *V. parahaemolyticus* is reported. The method included a set of three controls (process, hybridization and detection control) for the full monitoring of the analytical procedure. Four digoxigenin-labeled probes were designed for pathogenic strains enumeration (tdh1, tdh2, trh1 and trh2 probes) and one for total *V. parahaemolyticus* count (toxR probe). Probes were tested on a panel of 70 reference strains and 356 environmental, food and clinical isolates, determining the inclusivity (*tdh*: 96.7%, *trh*: 97.8%, *toxR*: 99.4%) and the exclusivity (100% for all probes). Accuracy and linearity of the enumeration were evaluated on pure and mixed cultures: slopes of the regression lines ranged from 0.957 to 1.058 depending on the target gene and $R^2$ was greater than or equal to 0.989 for all reactions. Evaluation was also carried on using four experimentally contaminated seafood matrices (shellfish, finfish, crustaceans and cephalopods) and the slopes of the curves varied from 0.895 (finfish) to 0.987 (cephalopods) for the counts of potentially pathogenic *V. parahaemolyticus* ($R^2 \geq 0.965$) and from 0.965 to 1.073 for total *V. parahaemolyticus* enumeration ($R^2 \geq 0.981$). Validation was performed on 104 naturally contaminated shellfish samples, analyzed in parallel by colony hybridization, ISO/TS 21872-1 and MPN enumeration. Colony hybridization and ISO method showed a relative accuracy of 86.7%, and a statistically significant correlation was present between colony hybridization enumeration and MPN results ($r = 0.744$, $p < 0.001$).

The proposed colony hybridization can be a suitable alternative method for the enumeration of total and potentially pathogenic *V. parahaemolyticus* in seafood.



## 1. Introduction

*Vibrio parahaemolyticus* is a halophilic microorganism naturally present in marine and estuarine environments worldwide, recognized as the causative agent of human gastroenteritis following the consumption of raw, inadequately cooked or mishandled seafood (Deepanjali et al., 2005; Gonzalez-Escalona et al., 2005; Mead et al., 1999). Molecular epidemiology studies showed strong correlation between the presence in *V. parahaemolyticus* genome of particular virulence factors and the ability to cause disease and strains carrying the *tdh* gene, encoding the thermostable direct hemolysin (TDH), and/or the *trh* gene, encoding the TDH-related hemolysin (TRH), are considered pathogenic to man (Nishibuchi and Kaper, 1995; Zhang and Austin, 2005). To date, five types of *tdh* genes with 96% to 98% DNA sequence similarity have been identified: with the exception of *tdh3* that is present on a plasmid, all the variants are chromosome-borne with TDH-producing strains usually harboring both *tdh1* and *tdh2* (Baba et al., 1991; Nishibuchi and Kaper, 1990). TDH production in such strains is mainly contributed by *tdh2* (Nishibuchi et al., 1991), while the other *tdh* genotypes, probably in relation to nucleotide changes within the promoter region, were shown to have very low level of gene expression (Okuda and Nishibuchi, 1998; Yoh et al., 1992). The *trh* genes present a higher strain to strain sequence variation and can be clustered into two main subgroups (*trh1* and *trh2*), which share 84% identity with each other (Kishishita et al., 1992) and approximately 68% with *tdh* sequences (Nishibuchi et al., 1989). The two variants usually do not coexist (Nakaguchi et al., 2004) and their distribution seems to indicate preponderance of *trh2* in environmental samples (Kishishita et al., 1992; Suffredini et al., 2011). Strains carrying both *tdh* and

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*trh* genes have also been detected and were found to produce TDH at much lower levels respect to isolates harboring only the *tdh* genes (Shirai et al., 1990; Suzuki et al., 1997). Overall, questions have been raised on the adaptation significance of these virulence marker in *V. parahaemolyticus* populations, as frequency of either *tdh* or *trh* gene in isolates from environmental samples and seafood has been reported to range from 0.3 to 15% of total *V. parahaemolyticus* (DePaola et al., 2003b; Deter et al., 2010; Hervio-Heath et al., 2002; Robert-Pillot et al., 2004), significantly lower than in clinical isolates (Cook et al., 2002; DePaola et al., 2003b; Hayat Mahmud et al., 2006).

Epidemiological data show that *V. parahaemolyticus* is an important cause of foodborne illness in in Asia, South America and the United States (Chiou et al., 2000; Fuenzalida et al., 2006; Gil et al., 2007; Haendiges et al., 2014; Ma et al., 2014; Okuda et al., 1997; Sims et al., 2011), while only a few outbreaks or sporadic cases are reported in Europe (Martinez-Urtaza et al., 2004; Ottaviani et al., 2010; Quilici et al., 2005). However, although *Vibrio* risk appears to be low in Europe, international experience demonstrated that outbreaks can occur unexpectedly, in relation, among other reasons, with the spread of highly virulent strains (Martinez-Urtaza et al., 2013; Okuda et al., 1997) and concern has been expressed on a possible rise of human vibriosis due to the increase of seawater temperature (Baker-Austin et al., 2013; Vezzulli et al., 2012). On the basis of this considerations and with the aim of collecting data on the real occurrence of pathogenic *V. parahaemolyticus* strains in seafood circulating in the Member States, the EU Regulation recommends the development of an effective standardized method for the detection of this micro-organism in seafood products.

Currently a cultural standard method (ISO/TS 21872-1:2007/Cor. 1:2008) is available for *V. parahaemolyticus* detection and many molecular methods for the rapid detection of *V. parahaemolyticus* have been developed, based on conventional PCR (Hossain et al., 2013; Rosec et al., 2012), real-time PCR (Kim et al., 2008; Liu et al., 2012; Tyagi et al., 2009; Ward and Bej, 2006), LAMP (Wang et al., 2013; Yamazaki et al., 2011), microarray (Wang et al., 2011) or combined molecular–immunological assay (Di Pinto et al., 2012). Quantitative analysis of *V. parahaemolyticus*, which is essential to accomplish risk assessment purposes, has also been attempted through real time q-PCR (Cai et al., 2006; Nordstrom et al., 2007), but the presence of inhibitors has hampered the achieving of acceptable quantification limits in food matrices, directing studies to the application of MPN-PCR enumeration techniques (Copin et al., 2012; Croci et al., 2002; Jones et al., 2012; Robert-Pillot et al., 2010).

An alternative approach to the detection and characterization of *V. parahaemolyticus*, based on the use of colony hybridization with nonisotopic probes, has been proposed by different authors (Lee et al., 1995; McCarthy et al., 1999; Nishibuchi et al., 1985; Numata et al., 2000). Colony hybridization technique was shown to reduce length of analysis and increase the detection rate of *V. parahaemolyticus* when combined with enrichment steps (Nordstrom and DePaola, 2003) and its use for the enumeration of this microorganism has been reported in Bacteriological Analytical Manual (FDA, 2004) and extensively used in the US (DePaola et al., 2003a, 2009; Jones et al., 2009; Parveen et al., 2008).

The aim of the current study was the development of a robust DNA colony hybridization method for the enumeration of enteropathogenic and total *V. parahaemolyticus* in seafood. The development of the method took into account, besides the performance characteristics (inclusivity, exclusivity, enumeration accuracy, dynamic range, etc.), the application of adequate controls for each step of the procedure, as well as international availability and quality control of critical reagents. In-house validation of the method was performed for the shellfish matrix, using naturally contaminated samples and comparing the results with ISO/TS 21872-1:2007/Corr.1:2008 and conventional MPN enumeration.

## 2. Materials and methods

### 2.1. Bacterial strains

A total of 426 strains (Table 1), including 70 reference strains from the American Type Culture Collection (ATCC, Manassas, VA, USA), the Culture Collection of the University of Göteborg (CCUG, Göteborg, Sweden), the National Collection of Type Cultures (NCTC, London, UK) and the Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection (BCCM/LMG, Gent, Belgium) and 356 isolates from fishery products, environmental sources and clinical cases were used. Among these, 175 were *V. parahaemolyticus* (106 non-toxigenic and 69 *tdh* and/or *trh* positive strains), 215 represented other *Vibrio* species and 36 belonged to genera other than *Vibrio* (Appendix A, Table 1). Thirty three strains were kindly supplied by Dr. J. Martinez-Urtaza (Universidad de Santiago de Compostela) in the form of DNA linked to hybridization membranes; one strain (*V. alginolyticus* GCSLVa29) was supplied by Dr. A. DePaola (*FDA* Seafood Laboratories) as pure culture DNA extract.

All *Vibrio* isolates had been identified or characterized by PCR (Bej et al., 1999; Brauns et al., 1991; Dalmasso et al., 2009; Di Pinto et al., 2005; Kim et al., 1999; Nandi et al., 2000). The long term storage of the bacterial strains was performed at −80 °C in commercially available cryogenic vials (Microbank™, Prolab Diagnostic, Neston, UK).

### 2.2. Samples

Four seafood matrices, shellfish (*Mytilus galloprovincialis*), finfish (*Sparus aurata*), crustaceans (*Penaeus japonicus*) and cephalopods (*Octopus vulgaris*), were selected for tests on experimentally contaminated samples. Shellfish of different species (*M. galloprovincialis*, *Tapes decussatus*, *Tapes philippinarum*, *Venus gallina*, *Crassostrea gigas*, *Callista chione*, *Cardium* sp., *Solen* sp., *Chlamys* sp., *Murex* sp.) were used for validation of the method on naturally contaminated products. Samples were purchased from local markets or were collected directly in authorized shellfish production areas of the Regions Lazio, Sardinia and Veneto (Italy).

Samples were shipped refrigerated to the laboratory and analyzed upon arrival. For each sample 50 g of flesh and, for shellfish samples, intravalvular fluid were taken, diluted 1:1 (wt/wt) with peptone-salt solution (Oxoid, Basingstoke, UK) and homogenized in a rotary blender for 90–120 s (Fig. 1). Tenfold dilutions were prepared and a 0.2 g aliquot of the homogenate (equivalent to 0.1 g of food sample) or 100 μl portions from the subsequent dilutions were spread onto a saline tryptone soya agar (TSA-S, Oxoid) plates and incubated at 37 ± 1 °C for 18–24 h.

### 2.3. Method development

#### 2.3.1. Probe design

Digoxigenin-labeled probes were designed for the *toxR* gene of *V. parahaemolyticus* (enumeration of total *V. parahaemolyticus*) and for the genes *tdh1*, *tdh2*, *trh1* and *trh2* (enumeration of potentially pathogenic *V. parahaemolyticus*).

For probe design a total of 134 sequences for *toxR* (n = 88), *tdh* (n = 43), *trh* (n = 21) genes of *Vibrio* and other marine environmental species (e.g. *Pseudomonas* spp., *Aeromonas* spp., *Photobacterium* spp., etc.) were downloaded from international databases or obtained by in-house sequencing of PCR amplified products (Appendix A, Table 2). Sequences were separately aligned using the CLUSTAL W software v. 1.4 (Thompson et al., 1994) and analyzed with the BioEdit software v. 5.0.9 (Hall, 1999) for the detection of *V. parahaemolyticus* species-specific conserved zones. Probes were manually designed using CG content (40 to 45%) and basic melting temperature (60 to 70 °C) as acceptability criteria and were subjected to BLAST search to determine