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Evaluation of alkaline phosphatase- and digoxigenin-labelled probes for detection of the thermolabile hemolysin (*tlh*) gene of *Vibrio parahaemolyticus*

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S.A. McCARTHY, A. DePAOLA, D.W. COOK, C.A. KAYSNER AND W.E. HILL. 1999. The biochemical identification and enumeration of *Vibrio parahaemolyticus* as described in the FDA *Bacteriological Analytical Manual* is expensive and labour-intensive. To reduce the time and effort necessary to verify the identity of *V. parahaemolyticus*, the use of a thermolabile haemolysin (*tlh*) gene probe is proposed. An alkaline phosphatase (AP)-labelled probe was evaluated for specificity against 26 strains of *V. parahaemolyticus*, 88 strains of other *Vibrio* species and 10 strains of non-vibrio species. Of the 124 isolates tested, the probe hybridized only with the 26 strains of *V. parahaemolyticus,* indicating species specificity. Two hundred and six suspect *V. parahaemolyticus* isolates from oysters were tested by this probe and API-20E diagnostic strips; there was 97% agreement between results. A digoxigenin (DIG)-labelled probe for detection of the *tlh* gene fragment was prepared by PCR and compared with the AP-labelled probe. When tested on 584 suspect *V. parahaemolyticus* isolates, results obtained with the AP- and DIG-labelled probes were in 98% agreement. These results suggest that the probes are equivalent for detection of the *V. parahaemolyticus tlh* gene.

INTRODUCTION

Vibrio parahaemolyticus is a halophilic bacterium often found within shellfish and in estuarine waters. Among a number of pathogenic vibrio species, it is commonly associated with oyster-borne gastroenteritis (Rippey 1994). The organism was first described after an outbreak of food poisoning in Osaka, Japan, in 1950 (Fujino *et al*. 1951).

Vibrio parahaemolyticus strains produce a thermolabile direct haemolysin (TLH) that is species-specific (Taniguchi *et al*. 1985). Most clinical isolates also produce a thermostable direct haemolysin (TDH) that is associated with the Kanagawa phenomenon (production of beta-haemolysis on Wagatsuma blood agar) (Honda *et al*. 1988; Nishibuchi and Kaper 1995). Certain Kanagawa phenomenon-negative strains have also been reported to produce a TDH-related haemolysin (TRH) (Honda *et al.* 1988). Both TDH-positive strains and TRH-positive strains of *V. parahaemolyticus* are strongly associated with gastroenteritis. The *tdh* gene is also present in all strains of *V. hollisae* and some strains of *V. mimicus* and *V. cholerae* non-O1 (Nishibuchi and Kaper 1995). However, most non-pathogenic *V. parahaemolyticus* strains isolated from foods and the environment have neither *tdh* nor *trh* genes (Shirai *et al*. 1990; Kishishita *et al.* 1992).

The United States Food and Drug Administration (FDA) established 10 000 *V. parahaemolyticus* cells g^{-1} as a level that warrants regulatory action for ready-to-eat seafood products. This includes pathogenic (Kanagawa phenomenon-positive) and non-pathogenic (Kanagawa phenomenon-negative) strains, both of which must be enumerated in these foods (DHHS 1995, 1996). The most commonly accepted method for detection of *V. parahaemolyticus* in foods is described in the FDA *Bacteriological Analytical Manual* (BAM) (Elliot *et al*. 1995). The procedure involves estimation of the most probable number (MPN) and is recommended by the American Public Health Association (Miescier *et al*. 1992). Identification of *V. parahaemolyticus* by this method takes at least 4 d and subsequent biochemical identification of isolates is laborious and expensive. The MPN estimation requires screening of numerous isolates because of the abundance of

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V. parahaemolyticus in shellfish; this is a major drawback of this method. The use of a probe for identification of *V. parahaemolyticus* would reduce this testing period from a minimum of 4 d to 3 d. Radioactive and non-isotopic probes have been developed to identify pathogenic *V. parahaemolyticus tdh* and *trh* genes (Nishibuchi *et al*. 1985; Yamamoto *et al.* 1991; Lee *et al*. 1992). Non-isotopic probes are preferable because radioactive probes are difficult to handle and are unacceptable for routine use (Yamamoto *et al*. 1991). The purpose of this study was to evaluate a non-isotopic species-specific probe that is safe to handle and that can be used to verify suspect isolates from seafood products as *V. parahaemolyticus*.

MATERIALS AND METHODS

Bacterial strains

Eighty-four of the isolates used in this study were from environmental (water, sediment, seafood products) and clinical (patient) sources; 40 isolates were from unknown sources. Several of the strains were provided by E. Elliot and F. Khambaty, FDA, Center for Food Safety and Applied Nutrition (CFSAN, Washington, D.C., USA) and W. Landry, FDA, Dallas District Office (DDO, Dallas, TX, USA). The remainder were from the FDA, Gulf Coast Seafood Laboratory (GCSL, Dauphin Island, AL) and FDA, Seafood Products Research Center (SPRC, Bothell, WA) culture collections. A list of the isolates used in this study is given in Table 1. The isolates included 26 *V. parahaemolyticus*, 13 *V. vulnificus*, 26 *V. cholerae* non-O1; seven *V. cholerae* O1, 12 *V. fluvialis*, 13 *V. mimicus*, nine *V. hollisae*, two *V. alginolyticus*, three *V. damsela,* one *V. furnissii* (CDC1955–83), one *V. metschnikovii* (ATCC7708) and one *V. anguillarum* (ATCC19264). Other isolates examined were four *Aeromonas hydrophila*, two *Salmonella* spp., two *Escherichia coli* and two *Bacillus subtilis* (ATCC strains 23857 and 33234). The *B. subtilis* strains were used because of their similarity to the *V. parahaemolyticus tlh* gene as determined by a FASTA search (Pearson and Lipman 1988) in conjunction with the Genetics Computer Group software ver. 8 (Devereux *et al*. 1984). Cultures were maintained at room temperature on T1N1 agar slants (1% tryptone (Difco), 1% NaCl and 2% agar) overlaid with sterile mineral oil, or were inoculated using sterile toothpicks into 96-well plates (Costar, Cambridge, MA, USA) containing 100 μl well^{-1} alkaline peptone water (APW, 1% peptone (Difco) and 1% NaCl, pH 8·5). Cultures in 96-well plates were incubated at 35 °C for 18 h, amended with 100 μl well^{-1} cryoprotectant (24% glycerol (Sigma) in tryptic soy broth (TSB, Difco)) and stored at −80 °C.

Table 1 Bacterial strains used in study

Organism	No. of strains	Source
Vibrio parahaemolyticus	15	Env
	11	Clin
Vibrio vulnificus	13	Env
Vibrio cholerae non-O1	10	Env
	3	Clin
	13	Unk
Vibrio cholerae O1	1	Env
	6	Clin
Vibrio fluvialis	4	Env
	8	Unk
Vibrio mimicus	8	Env
	5	Unk
Vibrio hollisae	4	Clin
	5	Unk
Vibrio alginolyticus	2	Env
Vibrio damsela	3	Env
Vibrio furnissii (CDC1955-83)	1	Unk
Vibrio metschnikovii (ATCC7708)	1	Unk
Vibrio anguillarum (ATCC19264)	1	Unk
Aeromonas hydrophila	4	Env
Salmonella spp.	2	Unk
Escherichia coli	2	Unk
Bacillus subtilis (ATCC23857, ATCC33234)	2	Unk

Env, environmental source; Clin, clinical source; Unk, unknown source; CDC, Centers for Disease Control and Prevention (Atlanta, GA); ATCC, American Type Culture Collection (Rockville, MD).

Culture preparation

Bacterial colonies were transferred from agar media using sterile toothpicks to 96-well plates containing 100 μl well^{-1} APW and incubated at 35 °C for 18 h. Growth in wells was transferred to T1N3 agar plates (1% tryptone, 3% NaCl and 2% agar) using a flame-sterilized 48-prong inoculating block, with incubation at 35 °C, 18 h. Unless otherwise stated, all media and equipment were sterilized by autoclaving for 15 min at 121 °C.

Detection of *Vibrio parahaemolyticus* by gene probe

Alkaline phosphatase-labelled probe. The alkaline phosphatase (AP)-conjugated oligonucleotide probe was prepared by DNA Technology A/S (DK-8000 Aarhus C., Denmark) using a proprietary process. The nucleotide base sequence for the probe was from bases 904–927 of the *V. parahaemolyticus tlh* gene (accession number M36437) (Taniguchi *et al*. 1986; Brasher *et al*. 1998). The sequence for the *tlh*

probe was 5′-XAA AGC GGA TTA TGC AGA AGC ACT G-3′; X denotes alkaline phosphatase conjugated 5′ amine-C6. Filter preparation, hybridization and colorimetric detection were done as described by Wright *et al.* (1996) for *V. vulnificus,* except that hybridization was done at 54 °C.

Digoxigenin-labelled probe. The digoxigenin (DIG)-labelled probe (450 bases) was synthesized by polymerase chain reaction (PCR) (Brasher *et al.* 1998). Probe and filter preparation, hybridization and colorimetric detection were done according to *The Genius System User's Guide for Filter Hybridization*, Version 2·0 9–92 (Boehringer Mannhein Corp. 1992).

Analyses of oysters

Pacific oysters (*Crassostrea gigas*) were collected from representative growing areas and retail markets in Washington and Oregon states during an outbreak investigation from July to September 1997. Twelve live oysters or shucked oyster product were cooled in an ice chest with packaged ice, transported to the FDA District Laboratory in Bothell, WA, and analysed within 24 h of collection. Thirty-five samples, including four live samples from Oregon and four of shucked oyster product from Washington, were analysed by the FDA BAM method (Elliot *et al.* 1995) using a three tube, multiple dilution MPN procedure with enrichment in APW and isolation on thiosulphate-citrate-bile salts-sucrose (TCBS) agar plates. Two or three sucrose-negative (green) colonies were selected from each plate for screening, using the arginine reaction on arginine-glucose slants (AGS) and growth in tryptone with 3% NaCl.

Gulf Coast oysters (*Crassostrea virginica*) were harvested by commercial means from the Mississippi Sound area of Alabama and held in tanks with flowing sea water (25 ppt, 27–29 °C) at the Gulf Coast Seafood Laboratory. Oysters (12) were shucked and homogenized without diluent for 90 s in a Waring blender and analysed as described above. The oysters were analysed (a) within 30 min of removal from the tanks (harvest), (b) after being held at outdoor ambient air temperature (25–29 °C) in the shade for 24 h (abused), and (c) after being abused and then held at 4 °C for 8 or 15 d in order to determine the effects of time and temperature on survival/growth of *V. parahaemolyticus.*

Sucrose-negative colonies were inoculated using sterile tooth picks into 96-well plates containing 100 μl APW well^{-1}. After 4 h incubation at 35 °C, cultures were replicated using a 48-prong replica plating block to *V. vulnificus* agar (VVA) (Wright *et al.* 1993) for colony lifts with Whatman 541 filters and to nylon membrane filters on VVA. Filters were hybridized, respectively, with AP- and DIG-labelled probes for the *tlh* gene. Each isolate was identified using API-20E diagnostic strips (bioMerieux).

RESULTS AND DISCUSSION

Specificity of alkaline phosphatase-conjugated *tlh* probe

The AP-conjugated *tlh* probe was hybridized to DNA from 26 previously characterized *V. parahaemolyticus* isolates from clinical and environmental sources. The probe hybridized with all 26 strains and verified the presence of the *tlh* gene. Several other *Vibrio* spp., i.e. *V. vulnificus*, *V. cholerae* non-O1, *V. cholerae* O1, *V. fluvialis*, *V. mimicus*, *V. hollisae*, *V. alginolyticus*, *V. damsela*, *V. furnissii*, *V. metschnikovii* and *V. anguillarum* were used to test cross-hybridization of the *tlh* probe. None of these 88 strains of vibrio hybridized with the *tlh* probe. Ten strains of *Aer. hydrophila*, *Salmonella* spp., *E. coli* and *B. subtilis* were also tested for cross-hybridization, and none was observed. These results demonstrated the species specificity of this oligonucleotide.

Table 2 Identification of *Vibrio parahaemolyticus* by alkaline phosphatase-labelled *tlh* gene probe and by API-20E

Source	Treatment	No. tested	Probe + API +	Probe − API −	Probe + API −	Probe − API +
Pacific NW oysters	Harvest	126	126	0	0	0
Gulf Coast oysters	Harvest	25	15	7	2	1
Gulf Coast oysters	Abused	22	16	4	1	1
Gulf Coast oysters	4 °C, 3 d	14	12	2	0	0
Gulf Coast oysters	4 °C, 15 d	19	18	0	1	0
Total		206	187	13	4	2

Harvest, oysters analysed within 30 min of removal from tank; abused, oysters held in shade at 25–29 °C, 24 h; 4 °C, oysters abused and then held at 4 °C for 8 and 15 d; (+), identified as *V. parahaemolyticus*; (−), not identified as *V. parahaemolyticus*.

Table 3 Reaction of alkaline phosphatase (AP)- and digoxigenin (DIG)-labelled *tlh* gene probes with suspect isolates from oysters

Source	No. tested	AP+/DIG+	AP−/DIG−	AP+/DIG−	AP−/DIG+
Pacific NW oysters	319	319	0	0	0
Gulf Coast oysters	265	203	51	10	1

+, Identified as *V. parahaemolyticus*; −, not identified as *V. parahaemolyticus.*

Vibrio isolates from Gulf Coast and Pacific oysters

Sucrose-negative vibrio isolates (206) from Gulf Coast and Pacific oysters were tested with the AP-labelled *tlh* probe and by API-20E diagnostic strips (Table 2). Results obtained by both tests were in agreement for 97% of the isolates.

Both methods identified all (126) sucrose-negative suspects from Pacific oysters as *V. parahaemolyticus*. The sucrose-negative microflora from Gulf Coast oysters was more diverse; only 60% of isolates from oysters at harvest (oysters analysed within 30 min of removal from tank) were identified as *V. parahaemolyticus* by both methods. Other sucrose-negative organisms could include *V. vulnificus*, *V. mimicus*, or certain species of *Pseudomonas*, *Aeromonas*, *Flavobacterium* or *Alteromonas.*

Numbers of *V. parahaemolyticus* in Gulf Coast oysters increased after abuse (25–29 °C, 24 h) plus refrigerated storage (4 °C, 8 and 15 d) to approximately 90% of the sucrose-negative population. Overall, after time and temperature abuse of Gulf Coast oysters, the AP-labelled probe and API-20E test results agreed for 92·5% of the isolates (Table 2).

Five hundred and eighty-four vibrio isolates from Pacific Northwest and Gulf Coast oysters were tested with the AP- and DIG-labelled probes (Table 3). Identification of *V. parahaemolyticus* based on detection of the *tlh* gene by both probes was in 97·9% agreement.

The AP-labelled probe offers the advantage of a 1 h hybridization time, thus allowing completion of the analysis within one working day for colonies lifted from the VVA plates. One lot of commercially labelled *tlh* probe (0·2 μmol) is enough to process about 500 filters. The DIG-labelled probe can be prepared in-house, is less expensive and contains more reporter groups per probe molecule. The probe solution can be used several times and the hybridization and wash temperatures are the same for all probes. The results suggest that the AP- and DIG-labelled *tlh* gene probes are equivalent and that either probe technique could be used in lieu of biochemical tests. On the basis of current costs, this would reduce the expense of labour and reagents by 80–90%. The *tlh* gene probes can identify the presence of both pathogenic and non-pathogenic strains of *V. parahaemolyticus* in a sample without distinguishing the purported virulence genes, *tdh* and *trh,* of pathogenic strains.

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