FOOD BIOLOGICAL CONTAMINANTS

Detection, Enumeration, and Isolation of *Vibrio parahaemolyticus* and *V. vulnificus* from Seafood: Development of a Multidisciplinary Protocol

Swapan K. Banerjee
Health Canada, Health Products and Food Branch, Bureau of Microbial Hazards, 251 Sir Frederick Banting Driveway, Ottawa, ON K1A 0K9, Canada
Jeffrey M. Farber
Health Canada, Health Products and Food Branch, Bureau of Microbial Hazards, 251 Sir Frederick Banting Driveway, Ottawa, ON K1A 0K9, Canada; University of Guelph, Department of Food Science, Bldg 38, Guelph, ON N1G 2W1, Canada

***Vibrio parahaemolyticus* and *V. vulnificus* are bacterial foodborne pathogens that can cause illnesses in humans after ingestion or exposure to contaminated seafood or coastal waters. A procedure that combines microbiological, biochemical, and molecular methods was designed and optimized for the detection, enumeration, isolation, and characterization of these clinically significant *Vibrio* spp. Initially, microbiological culturing is used to resuscitate and isolate presumptive *Vibrio* spp. from chilled seafood samples. Biochemical tests are then used to analyze and select presumptive isolates at the species level, and, lastly, molecular methods, such as PCR targeting species-specific hemolysin genes, are used to confirm identification and assess the potential pathogenicity of presumptive isolates. By using artificially contaminated molluscan homogenates with known numbers of *V. parahaemolyticus*, this method yielded, on average, 90% recovery on complete agar media and 88% recovery on selective media. For *V. vulnificus*, the recovery rates were 86% (complete media) and 84% (selective media). Linearity of recovery of *Vibrio* spp. from artificially contaminated seafood homogenates supported the applicability of this method. Overall, this performance-tested protocol is easy to use, cost-effective, and fit-for-purpose, with potential for routine use in basic microbiological facilities.**

Vibrio spp. are rod-shaped Gram-negative bacteria that are widespread in coastal and estuarine environments around the world. The halophilic biovars of clinical significance, *Vibrio parahaemolyticus* and *V. vulnificus*, require moderate to high saline environments to survive and often colonize marine animals, notably invertebrates. Illnesses in humans occur after ingestion or direct wound contact with contaminated seafood or seawater (1–4).

V. parahaemolyticus was first identified as a foodborne pathogen in the 1950s, and, in subsequent decades, became globally recognized as an etiological agent of diarrheal disease associated with seafood consumption (5, 6). Virulence factors of *V. parahaemolyticus* include proteases, secretion systems, adhesins, the expression of toxins encoded by the *toxR* operons, and, most notably, the hemolysin genes: thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*; 7, 8). Another hemolysin gene, thermolabile hemolysin (*tlh*), is detected in all *V. parahaemolyticus* (7, 9). *V. vulnificus* has been associated with a small but increasing number of serious life-threatening conditions, such as septicemia, which may lead to hypotension, shock, and eventually death unless timely intervention with antibiotics is received (10, 11). In the United States, *V. vulnificus* has been identified as being responsible for most of the seafood-related deaths since the first report of it in 1979 (12). A regular source of infection with the pathogen is the consumption of contaminated raw or undercooked seafood, notably mollusks (10). Because of the serious nature of human disease attributed to *V. vulnificus*, a great deal of attention has focused on understanding its pathogenicity mechanisms. It has been determined that isolates produce a range of pathogenicity factors, including a polysaccharide capsule, hemolysin, type IV pili, and various proteases (e.g., a serine protease and a metalloprotease; 13).

The source of most seafood-borne pathogens is the water and sediments from which the seafood is harvested. The distribution of the total coastal *V. parahaemolyticus* population is influenced by environmental factors, including temperature, turbidity, salinity (14, 15), and factors related to plankton (16). Higher levels of pathogenic *V. parahaemolyticus* are reportedly emerging in the (cold) temperate regions of the world (4, 6, 17, 18), coinciding with the events of climate change and increasing global trade. Similarly, climate, especially rising temperatures, has been shown to increase the prevalence and natural range of *V. vulnificus* and even *V. cholerae*, the etiological agent of cholera (19–21).

The involvement of *Vibrio* spp. in human disease has prompted awareness and subsequent surveillance work for the purposes of developing science-based regulations. The trend of increased surveillance is expected to continue as climate change increases the range of these species and as population dynamics of this genus are changed by the horizontal exchange of traits, including virulence. The emerging trends observed through surveillance studies will provide evidence to design food safety regulations and policy in favor of disease control strategies.

Globally, seafood is produced in surplus from some developing countries and exported to developed countries where consumption has surpassed production. One of the major gaps identified by the Food and Agriculture Organization/World Health Organization (22) is the availability of sufficient data for

Received September 02, 2016. Accepted by AH October 13, 2016.
Corresponding author's e-mail: swapan.banerjee@hc-sc.gc.ca
DOI: 10.5740/jaoacint.16-0290

risk assessment regarding the regional prevalence of pathogenic *V. parahaemolyticus*, including its estimated abundance. Therefore, a method for the isolation and characterization of *V. parahaemolyticus* and *V. vulnificus* that is simple, cost-effective, reliable, and usable in any basic microbiological facility in the world is desirable.

V. parahaemolyticus and *V. vulnificus* can be differentiated on the basis of cellobiose fermentation because *V. vulnificus* can ferment this sugar and *V. parahaemolyticus* cannot. However, biochemical-based procedures do not provide reliable indications of potential pathogenicity. A published comparative study of methods for the identification of *V. parahaemolyticus* has suggested that all biochemical identifications should be confirmed by means of molecular methods (23). At the molecular level, the species-specific hemolysin genes *tlh*, *tdh*, and *trh* in *V. parahaemolyticus*, and *vvhA* (formerly known as *cth*; 7) in *V. vulnificus*, can be exploited to differentiate these species and predict pathogenicity. Therefore, we propose that (*1*) molecular-based diagnostic tests be performed to confirm and strengthen the identification and (*2*) an agreement between the multiple analyses done on microbiologically selected presumptive isolates can be accepted as confirmation of the species, as described in our protocol, which has been tested for more than a decade.

Materials and Methods

Bacterial Strains and Media

Reference strains of *Vibrio* spp. and other bacterial species were obtained from our inventory (9, 24), as well as from other sources [American Type Culture Collection (ATCC) and in-house culture collections]. Fifty *V. parahaemolyticus* strains, including ATCC 17802, NY477, and 48 other characterized clinical isolates (24), and 24 *V. vulnificus* strains, including ATCC 27562, C7184, and other characterized strains from our inventory, were selected for the required analyses. Nontargeted *Vibrio* spp., including *V. alginolyticus* (ATCC 17749), *V. cholerae* (ATCC 25872 and ATCC 35971), *V. fluvialis* (ATCC 33809), *V. furnisii* (ATCC 35016), *V. mimicus* (ATCC 33655), and *V. hollisae* (ATCC 33564), were used for molecular exclusivity tests. Molecular exclusivity tests also included other available bacterial species, such as *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35150 and ATCC 25922), *Salmonella* Derby (ATCC 43845), *Listeria innocua* (ATCC 33090), *L. monocytogenes* (ATCC 13932), *Shigella sonnei* (ATCC 29930), *S. boydii* (ATCC 9207), *Klebsiella pneumoniae* (ATCC 10031 and ATCC 49472), *Aeromonas sobria* (ATCC 9071), *A. hydrophila* (ATCC 7966), and *Cronobacter sakazakii* (ATCC 29544).

Alkaline peptone water (APW, pH 8.5) was made with 1% Bacto peptone and 2% NaCl. Premixed tryptic soy agar (TSA; Difco Laboratories, Becton, Dickinson and Co., United States) was supplemented with 1.5% NaCl to prepare TSA-2N. Thiosulfate–citrate–bile salts–sucrose agar (TCBS; Oxoid Ltd, Hampshire, England) was purchased as a premixed powder and prepared according to the manufacturer's instructions. Modified cellobiose–colistin (mCC) contained peptone (10 g L^{-1}), beef extract (5 g L^{-1}), NaCl (20 g L^{-1}), agar (15 g L^{-1}), cellobiose (10 g L^{-1}), 0.004% of each of Bromothymol Blue and Cresol Red dissolved in ethanol, and either 10 000 U (mCC10) or 400 000 U (mCC400) of colistin methanesulphonate (25, 26). CHROMagar Vibrio (CAV; CHROMagar, France) was prepared as per the manufacturer's instructions and other reports (27, 28). Storage agar slants (pH 8.5) were prepared using beef extract (5 g L^{-1}), peptone (10 g L^{-1}), NaCl (10 g L^{-1}), dibasic sodium phosphate (0.793 g L^{-1}), and agar (10 g L^{-1}).

Analytical Procedure

Various standard procedures and published methods were combined and used in the design of this stand-alone protocol, as outlined in Figure 1. Mollusks, including oysters, clams, and mussels were obtained from Canadian harvest sites and processed immediately for detection and enumeration analyses. Mollusks from retail outlets can be stored refrigerated or frozen to mimic the conditions at retail, and processed within 2 to 3 days. The protocol was characterized by testing performance efficiency determinants, as described in the *Performance Testing* section. Briefly, 10–20 mollusks were shucked and homogenized in a blender to obtain about 100–200 g smooth tissue, of which 50 g was mixed with 450 mL APW and then equilibrated at room temperature (approximately 23°C) for 60–75 min to resuscitate bacterial function. A 0.1 mL aliquot of culture was used for aerobic plate counts via direct plating (DP) on TCBS, mCC10, and CAV media to measure approximate levels of the targeted pathogens before enrichment, by using differential specificity of the selective cultures (Table 1) to eliminate confounding numbers of false positives in the estimation, particularly for enumerating *V. vulnificus* (27, 29). An aliquot can also be spread on TSA-2N (optional) to get an estimate of the total bacterial count. The APW containing sample homogenates was enriched overnight at

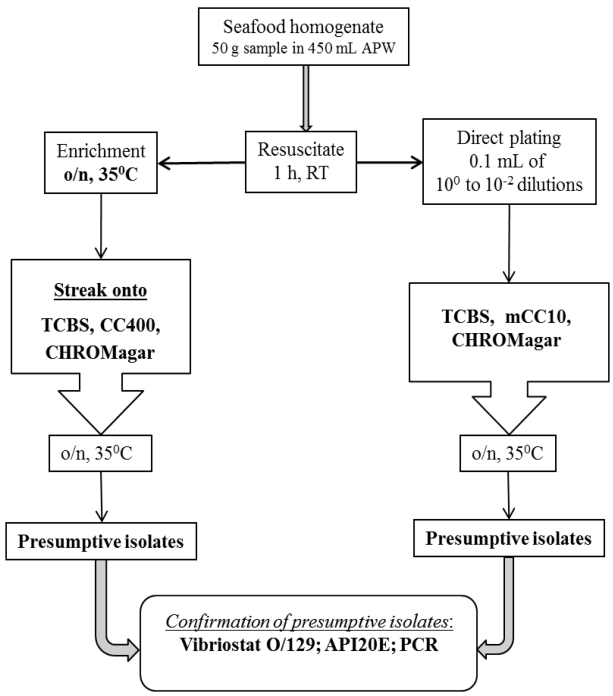

Figure 1. Flowchart demonstrating the steps used in the method, including resuscitation, selective isolation, and characterization of presumptive isolates of *V. parahaemolyticus* and *V. vulnificus* from seafood.

Table 1. Comparison of the phenotypic profiles of clinically important *Vibrio* spp. on selective growth media

	Colony phenotype on selective media		
Species	TCBS	CAV	mCC400
V. parahaemolyticus	Green	Mauve	NG/neutral[a]
V. vulnificus	Green	Blue	Yellow with halo
V. alginolyticus	Yellow	Cream	Yellow
V. cholerae	Yellow	Blue	Purple

[a] NG = No growth.

35°C. The cultures (10 µL) were streaked onto a combination of selective media (TCBS, mCC400, and CAV) for further isolation. Presumptive isolates were picked from selective plates after both DP and streaking of enriched cultures by comparing them with isolated colonies from similar streaks using standard strains of the targeted *Vibrio* spp. such as *V. parahaemolyticus* (ATCC 17802 or NY477) and *V. vulnificus* (ATCC 27562 or C7184). In some cases, the presumptive isolates were rechecked and partially confirmed by streaking onto complementary selective plates with differential specificity (Table 1). This strategy of double- or triple-plating selection increased confidence in the presumptive identification of the isolates and in the enumeration of contaminating *V. parahaemolyticus* or *V. vulnificus* in seafood. Presumptive isolates obtained from each of the samples were stored on agar slants for further characterization with biochemical and molecular approaches.

Each presumptive isolate was subjected to the rapid biochemical tests in API 20E diagnostic strips (bioMérieux Canada, Inc., Saint-Laurent, QC, Canada); this process was used to confirm the identity of selected presumptive isolates as either *V. vulnificus*, *V. parahaemolyticus*, or as another *Vibrio* sp. Briefly, each presumptive colony was taken from a fresh TSA-2N plate and resuspended in 5 mL saline (0.85% NaCl) and sometimes repeated by resuspending in 2% NaCl for better identification of *V. vulnificus* using biochemical API 20E diagnostic strips (30) following the procedure described by the manufacturer. In addition to the core API 20E tests, an oxidase test and a vibriostat test (O/129; 2,4-diamino-6, 7-diisopropylpteridine; Oxoid Ltd) were performed. For the oxidase test, isolated colonies from TSA-2N were picked and smeared directly onto the reaction area of the BBL DrySlide Oxidase (Becton Dickinson Microbiology Systems, Sparks, MD), as described by the manufacturer. A dark purple color within 30 s indicated a positive reaction. The sensitivity of *V. parahaemolyticus* isolates to the vibriostatic agent O/129 was determined by spreading uniform lawns of bacteria from the saline suspension onto TSA-2N using a cotton-tipped swab, and aseptically placing both 10 and 150 µg disks of O/129 on the plates. The plates were incubated at 35°C for at least 18 h, and a clear zone of inhibition of growth around the discs indicated susceptibility to O/129 at the observed concentration. *V. parahaemolyticus* is predominantly resistant to 10 µg O/129 but sensitive to a 150 µg concentration.

PCR Analysis

PCR testing completed the confirmation by identifying species-specific markers, including virulence markers for *V. parahaemolyticus* and *V. vulnificus*. Templates were prepared by boiling a portion (approximately 20%) of an isolated colony resuspended in 200 µL buffer solution (10 mM Tris and 0.1 mM EDTA) in a 1.5 mL microcentrifuge tube (mct) for 10 min, followed by a quick chill on ice and centrifugation to pellet the debris. The supernatant was stored at 4°C in a sterile mct, and a 2.5 µL aliquot was used each time for PCR analysis. Thermal cyclers (Bio-Rad, iCycler, or T100 Thermal Cycler) were programmed in accordance with the primer pairs used. Primers used to confirm the presence of *V. parahaemolyticus* were as follows: forward: 5′-AAA GCG GAT TAT GCA GAA GCA CTG-3′ and reverse: 5′-GCT ACT TTC TAG CAT TTT CTC TGC-3′, which generated a 450 bp amplicon of the *tlh* gene (7, 8, 26). To assess the pathogenicity of *V. parahaemolyticus*, primers that target the *tdh* and *trh* genes were used. The *tdh* primers (5′-GAA GTA CCG ATA TTT TGC-3′ and 5′-ATG TTG AAG CTG TAC TTG-3′) generated an amplicon of 385 bp (8, 26, 31). Primer pairs used for the detection of the *trh* gene were 5′-TTGGCTTCGATATTTTCAGTATCT-3′ and 5′-CATAACAAACATATGCCCATTTCCG-3′, which produced a 486 bp amplicon (8).

Appropriate reaction controls and generation of amplicons (positive control) were achieved by treating the standard strains ATCC 17802 ($tlh^+tdh^-trh^+$) and NY477 ($tlh^+tdh^+trh^-$) similarly, in parallel with the sample preparation, to rule out any PCR inhibition (process control). A reaction tube without any template (negative control) was added with each batch to test the reagents for the presence of contaminating DNA.

Primers used for detecting *V. vulnificus* were the following: forward: 5′-CGC CGC TCA CTG GGG CAG TGG CTG-3′ and reverse: 5′-CCA GCC GTT AAC CGA ACC ACC CGC-3′, which amplified a 388 bp section of the *vvhA* gene (32, 33). Identically prepared templates from the standard strain ATCC 27562 or C7184 were used as the positive and reaction (process) controls, in parallel with a negative (no template) control.

Performance Testing

Reference strains of *V. parahaemolyticus* and *V. vulnificus* were used for molecular inclusivity and exclusivity studies and in assessing the rates of recovery by artificially contaminating shellfish homogenates (Tables 2–6). For inclusivity testing of *V. parahaemolyticus*, known strains were streaked onto TCBS, and isolates that appeared green with a dark center and were 2 mm in diameter were picked as *V. parahaemolyticus*. These isolates were evaluated by using the primers targeting *tdh*, *trh*, and *tlh* genes to examine the inclusivity of this test. Similarly, *V. vulnificus* strains were reconfirmed as green colonies (approximately 2 mm in diameter) on TCBS and as flat yellow colonies with a yellow halo on mCC10, and selected as *V. vulnificus* for inclusivity using primers specific for the *vvhA* gene.

For exclusivity analysis, the strains listed in the *Bacterial Strains and Media* section were tested against each of the primer pairs to determine the exclusivity of these primer sets in differentiating *V. parahaemolyticus* or *V. vulnificus* from other bacterial isolates (Table 6).

Recovery of *V. parahaemolyticus* NY477 and *V. vulnificus* ATCC 27562 strains from artificially contaminated samples was tested by mixing a known number (1log–5log) of bacteria of the experimental strain with homogenized and irradiated (Co-60, gamma rays) mollusk tissues. Stationary phase

Table 2. Inclusivity test for *tlh* by PCR

Strain	*tlh*
V. parahaemolyticus NY477	Positive
V. parahaemolyticus ATCC 17802	Positive
V. parahaemolyticus D11	Positive
V. parahaemolyticus B113	Positive
V. parahaemolyticus 2269	Positive
V. parahaemolyticus T12739	Positive
V. parahaemolyticus T9109	Positive
V. parahaemolyticus H64024	Positive
V. parahaemolyticus F60004	Positive
V. parahaemolyticus H73608	Positive
V. parahaemolyticus T78315	Positive
V. parahaemolyticus W79972	Positive
V. parahaemolyticus W80709	Positive
V. parahaemolyticus H77953	Positive
V. parahaemolyticus M70100	Positive
V. parahaemolyticus M73593	Positive
V. parahaemolyticus T8994	Positive
V. parahaemolyticus F4395	Positive
V. parahaemolyticus M8293	Positive
V. parahaemolyticus F30368	Positive
V. parahaemolyticus F63267	Positive
V. parahaemolyticus A5Z924	Positive
V. parahaemolyticus A5Z988	Positive
V. parahaemolyticus A5Z1022	Positive
V. parahaemolyticus 09-4660	Positive
V. parahaemolyticus 09-4661	Positive
V. parahaemolyticus 09-4662	Positive
V. parahaemolyticus 09-4663	Positive
V. parahaemolyticus 09-4664	Positive
V. parahaemolyticus 09-4665	Positive
V. parahaemolyticus 09-4666	Positive
V. parahaemolyticus 09-4681	Positive
V. parahaemolyticus C140	Positive
V. parahaemolyticus C141	Positive
V. parahaemolyticus C142	Positive
V. parahaemolyticus C143	Positive
V. parahaemolyticus C144	Positive
V. parahaemolyticus C145	Positive
V. parahaemolyticus C146	Positive
V. parahaemolyticus C147	Positive
V. parahaemolyticus C149	Positive
V. parahaemolyticus C150	Positive
V. parahaemolyticus 07-2964	Positive
V. parahaemolyticus 07-2965	Positive
V. parahaemolyticus A4EZ700	Positive
V. parahaemolyticus A4EZ703	Positive
V. parahaemolyticus A4EZ724	Positive
V. parahaemolyticus A4EZ927	Positive
V. parahaemolyticus A4EZ964	Positive
V. parahaemolyticus A2EZ743	Positive

Table 3. Inclusivity test for *tdh* by PCR

Strain	*tdh*
V. parahaemolyticus NY477	Positive
V. parahaemolyticus ATCC 17802	Positive
V. parahaemolyticus A5Z273	Positive
V. parahaemolyticus A5Z652	Positive
V. parahaemolyticus A5Z853	Positive
V. parahaemolyticus A5Z860	Positive
V. parahaemolyticus A5Z878	Positive
V. parahaemolyticus A5Z905	Positive
V. parahaemolyticus A5Z924	Positive
V. parahaemolyticus A5Z988	Positive
V. parahaemolyticus A5Z1022	Positive
V. parahaemolyticus H64024	Positive
V. parahaemolyticus F60004	Positive
V. parahaemolyticus H73608	Positive
V. parahaemolyticus W80709	Positive
V. parahaemolyticus H77953	Positive
V. parahaemolyticus M70100	Positive
V. parahaemolyticus M73593	Positive
V. parahaemolyticus T8994	Positive
V. parahaemolyticus F4395	Positive
V. parahaemolyticus M8293	Positive
V. parahaemolyticus F30368	Positive
V. parahaemolyticus F63267	Positive
V. parahaemolyticus M48830	Positive
V. parahaemolyticus H11523	Positive
V. parahaemolyticus F1419	Positive
V. parahaemolyticus H18983	Positive
V. parahaemolyticus M59787	Positive
V. parahaemolyticus 09-4435	Positive
V. parahaemolyticus 09-4436	Positive
V. parahaemolyticus 09-3216	Positive
V. parahaemolyticus 09-3217	Positive
V. parahaemolyticus 09-3219	Positive
V. parahaemolyticus 08-0278	Positive
V. parahaemolyticus 07-1339	Positive
V. parahaemolyticus 07-2964	Positive
V. parahaemolyticus 07-2965	Positive
V. parahaemolyticus 05-3133	Positive
V. parahaemolyticus 05-4792	Positive
V. parahaemolyticus 04-1240	Positive
V. parahaemolyticus 04-2192	Positive
V. parahaemolyticus 04-2549	Positive
V. parahaemolyticus 04-2550	Positive
V. parahaemolyticus 04-2551	Positive
V. parahaemolyticus A4EZ724	Positive
V. parahaemolyticus A4EZ927	Positive
V. parahaemolyticus A4EZ964	Positive
V. parahaemolyticus A3EZ136	Positive
V. parahaemolyticus A3EZ634	Positive
V. parahaemolyticus A3EZ710	Positive

Table 4. Inclusivity test for *trh* by PCR

Strain	*trh*
V. parahaemolyticus 04-1290	Positive
V. parahaemolyticus 04-2192	Positive
V. parahaemolyticus 04-2550	Positive
V. parahaemolyticus 05-3133	Positive
V. parahaemolyticus 05-4792	Positive
V. parahaemolyticus 08-7626	Positive
V. parahaemolyticus 09-1772	Positive
V. parahaemolyticus 09-3216	Positive
V. parahaemolyticus 09-3217	Positive
V. parahaemolyticus 09-3218	Positive
V. parahaemolyticus 09-3219	Positive
V. parahaemolyticus 09-4434	Positive
V. parahaemolyticus 09-4436	Positive
V. parahaemolyticus 09-4660	Positive
V. parahaemolyticus 09-4661	Positive
V. parahaemolyticus 09-4662	Positive
V. parahaemolyticus 09-4663	Positive
V. parahaemolyticus 09-4664	Positive
V. parahaemolyticus 09-4665	Positive
V. parahaemolyticus 09-4666	Positive
V. parahaemolyticus 09-4681	Positive
V. parahaemolyticus H64024	Positive
V. parahaemolyticus F60004	Positive
V. parahaemolyticus H73608	Positive
V. parahaemolyticus T78315	Positive
V. parahaemolyticus W79972	Positive
V. parahaemolyticus W80709	Positive
V. parahaemolyticus H77953	Positive
V. parahaemolyticus M70100	Positive
V. parahaemolyticus M73593	Positive
V. parahaemolyticus T8994	Positive
V. parahaemolyticus F4395	Positive
V. parahaemolyticus W501	Positive
V. parahaemolyticus M48830	Positive
V. parahaemolyticus H11523	Positive
V. parahaemolyticus F1419	Positive
V. parahaemolyticus H18983	Positive
V. parahaemolyticus M59787	Positive
V. parahaemolyticus A5Z652	Positive
V. parahaemolyticus A5Z860	Positive
V. parahaemolyticus A5Z878	Positive
V. parahaemolyticus A5Z905	Positive
V. parahaemolyticus A5Z924	Positive
V. parahaemolyticus A5Z988	Positive
V. parahaemolyticus A5Z1022	Positive
V. parahaemolyticus A4EZ700	Positive
V. parahaemolyticus A4EZ703	Positive
V. parahaemolyticus A4EZ724	Positive
V. parahaemolyticus A4EZ964	Positive
V. parahaemolyticus A3EZ136	Positive

Table 5. Inclusivity test for *vvhA* by PCR

Strain	*vvhA*
V. vulnificus ATCC 27562	Positive
V. vulnificus LAM 624	Positive
V. vulnificus A1402	Positive
V. vulnificus ATCC 33147	Positive
V. vulnificus ATCC 33817	Positive
V. vulnificus BAA-87	Positive
V. vulnificus W108	Positive
V. vulnificus C7184	Positive
V. vulnificus FDA-QA-1	Positive
V. vulnificus FDA-QA-3	Positive
V. vulnificus ATCC 27562 (k)	Positive
V. vulnificus LAM 624 P1X	Positive
V. vulnificus LAM 624 P2X	Positive
V. vulnificus LAM 624 P3X	Positive
V. vulnificus LAM 624 P4X	Positive
V. vulnificus S272-10	Positive
V. vulnificus S272-12	Positive
V. vulnificus S286-4	Positive
V. vulnificus S286-12	Positive
V. vulnificus S286-26	Positive
V. vulnificus S302-5	Positive
V. vulnificus S302-6	Positive
V. vulnificus S302-7	Positive
V. vulnificus S333-16	Positive
V. vulnificus ATCC 33147	Positive
V. vulnificus ATCC 33817	Positive
V. vulnificus BAA-87	Positive

(overnight) cultures of the bacteria at several concentrations (100 µL 10-fold dilutions) were mixed with mollusk homogenates to achieve various initial inoculum levels (1log–5log) of artificial contamination, which were determined from DP enumeration of the (overnight) culture dilutions. Mixtures (spiked homogenates) were then refrigerated overnight (for 20–25 h) at 4°C to induce cold stress in the bacteria by simulating the storage conditions at retail outlets. We evaluated the rates of recovery of each of these strains, at various concentrations, from the representative seafood samples. The data coordinates were assessed for linearity by regression analysis, and average percentage recovery was calculated from the slope. Sensitivity of the procedure was also assessed by calculating the LOD for each of the cold-stressed *Vibrio* spp.

Results and Discussion

Seafood homogenates that were artificially inoculated with *V. parahaemolyticus* or *V. vulnificus* to simulate contaminated seafood were used to assess the protocol we developed in this study. The LOD of the inoculum after the resuscitation step was estimated to be as low as approximately 20 CFU/g from colony counts using DP onto five selective plates (Figure 2). After overnight enrichment, the LOD dropped to as low as 1 CFU/g. In the case of presumptive isolates obtained from the seafood samples, the diagnostic efficiency of the PCR-based

Table 6. Exclusivity tests by PCR

Strain name	*tlh*	*tdh*	*trh*	*vvhA*
V. parahaemolyticus NY477	NA[a]	NA	NA	Negative
V. parahaemolyticus ATCC 17802	NA	NA	NA	Negative
V. parahaemolyticus D11	NA	NA	NA	Negative
V. parahaemolyticus B113	NA	NA	NA	Negative
V. parahaemolyticus 2269	NA	NA	NA	Negative
V. parahaemolyticus H64024	NA	NA	NA	Negative
V. alginolyticus ATCC 17749	**Positive**[a]	Negative	Negative	Negative
V. vulnificus ATCC 27562	Negative	Negative	Negative	NA
V. vulnificus LAM 624	Negative	Negative	Negative	NA
V. vulnificus A1402	Negative	Negative	Negative	NA
V. vulnificus W108	Negative	Negative	Negative	NA
V. vulnificus C7184	Negative	Negative	Negative	NA
V. cholerae ATCC 25872	Negative	Negative	Negative	Negative
V. cholerae ATCC 35971	Negative	Negative	Negative	Negative
V. fluvialis ATCC 33809	Negative	Negative	Negative	Negative
V. furnisii ATCC 35016	Negative	Negative	Negative	Negative
V. mimicus ATCC 33655	Negative	Negative	Negative	Negative
V. hollisae ATCC 33564	Negative	Positive	Negative	Negative
S. aureus ATCC 25923	Negative	Negative	Negative	Negative
Bacillus cereus ATCC 14579	Negative	Negative	NA	Negative
P. aeruginosa ATCC 27853	Negative	Negative	Negative	Negative
E. coli ATCC 35150	Negative	Negative	Negative	**Positive**
E. coli ATCC 25922	Negative	Negative	Negative	Negative
S. Derby ATCC 43845	Negative	Negative	Negative	Negative
L. innocua ATCC 33090	Negative	Negative	Negative	Negative
L. monocytogenes ATCC 13932	Negative	Negative	Negative	Negative
S. sonnei ATCC 29930	Negative	Negative	Negative	Negative
S. flexneri 2b ATCC 12022	Negative	Negative	Negative	Negative
S. boydii 2 ATCC 25930	Negative	Negative	Negative	Negative
S. boydii 1 ATCC 9207	Negative	Negative	Negative	Negative
K. pneumoniae ATCC 10031	Negative	Negative	Negative	Negative
K. pneumoniae ATCC 49472	Negative	Negative	Negative	Negative
A. sobria ATCC 9071	Negative	Negative	Negative	Negative
A. hydrophila ATCC 7966	Negative	Negative	Negative	Negative
C. sakazakii ATCC 29544	Negative	Negative	Negative	Negative

[a] NA = Not applicable.

[b] Positive results are shown in bold type.

predictive result was high in comparison with the standard API 20E results. This multidisciplinary approach was found to be reliable; several trainees/researchers have used it in our laboratory during more than a decade to isolate and confirm the targeted species.

In the case of *V. parahaemolyticus* inclusivity analysis, amplicons were generated from the *tlh* primers using DNA extracted from 50 of 50 tested *V. parahaemolyticus* strains, indicating that this PCR procedure has a high specificity (Table 2). In addition, *tdh* was detected in 50 of 50 strains known to be positive for this gene (Table 3). The *trh* primer pair allowed detection in 50 of 50 strains known to harbor this gene (Table 4).

For *V. vulnificus* inclusivity testing, the primer pair specific for *vvhA* was able to produce an amplicon in 27 of 27 *V. vulnificus* strains, indicating the high sensitivity of this test (Table 5).

DNA from 29 characterized strains other than *V. parahaemolyticus* was used in a PCR with *tlh* primer pairs, of which 28 did not produce an amplicon but the *V. alginolyticus* strain ATCC 17749 did (Table 6). Each of the 29 strains was negative for amplification with the *tdh* and *trh* primer pairs. The *vvhA* primer pair failed to produce an amplicon with 28 of the strains other than *V. vulnificus*, although a weak (nonspecific) reaction was observed with *E. coli* ATCC 35150. This finding demonstrates a high specificity of the molecular tests used in this procedure.

Species specificity of the respective amplicons for both of the targeted *Vibrio* spp. was tested and confirmed earlier by selective Southern hybridization of the amplicons, with colonies immobilized on hydrophobic grid membrane filters (9).

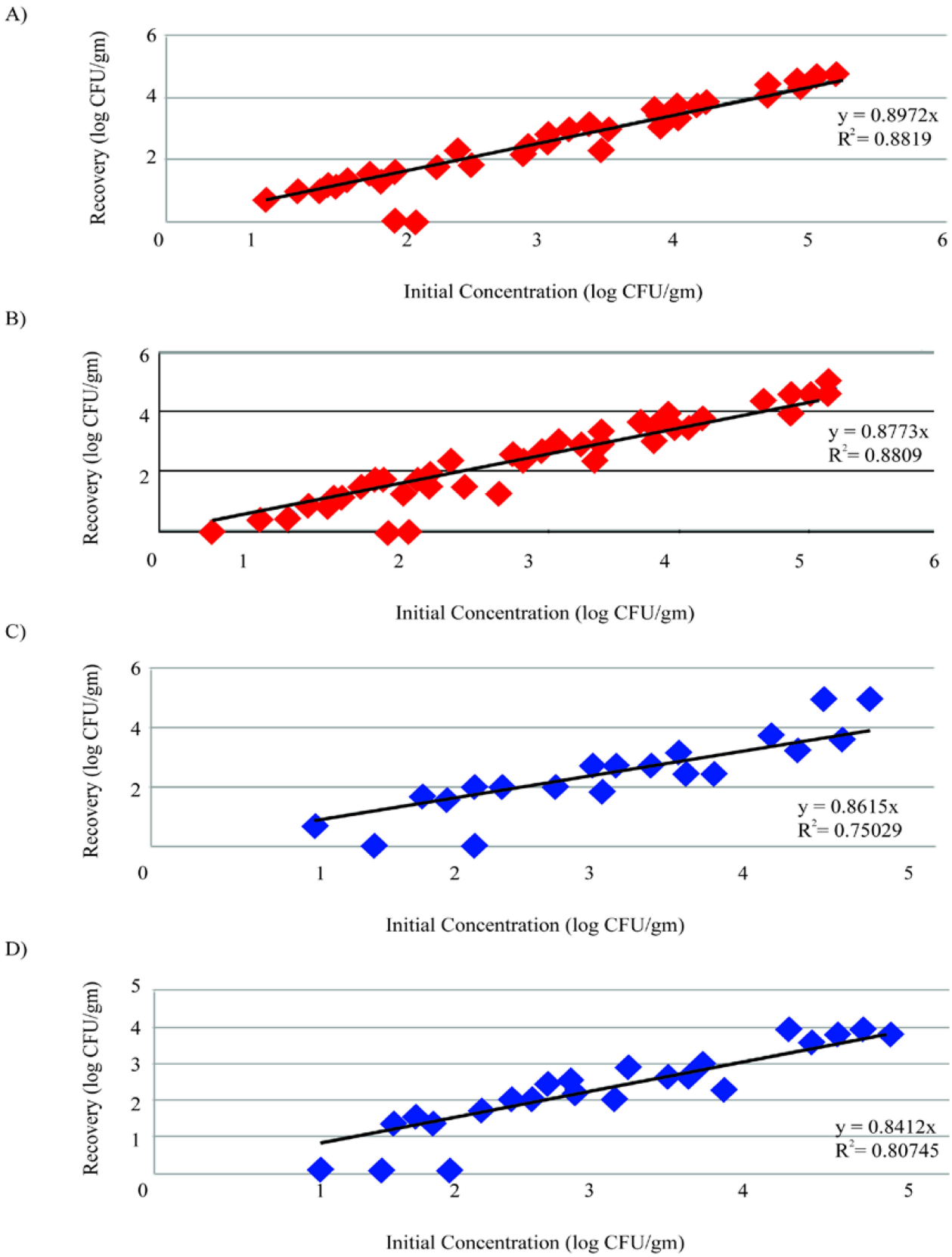

Figure 2. Linearity of recovery of the targeted species after artificial inoculation of mollusk homogenates prepared from oysters, clams, or mussels. Complete media and selective media for the respective species were used for determination of recovery. Recovery of *V. parahaemolyticus* NY477 using (A) TSA-2N and (B) TCBS plates. Recovery of *V. vulnificus* ATCC 27562 using (C) TSA-2N and (D) mCC10 plates.

For *V. parahaemolyticus* NY477, inoculations around 1log CFU/g of tissue were detected on TSA-2N and TCBS plates (Figure 2A and B), and this level (LOD) was reduced to as low as 1 CFU/g after overnight enrichment at 35°C. On average, the recovery of spiked CFU was calculated to be 90% on TSA-2N and 88% on TCBS plates after resuscitation and DP without enrichment (Figure 2A and B). The linear relationship was confirmed by regression analysis ($R^2 = 0.88$; TSA-2N or TCBS) when the samples were spiked with between 1log and 5log CFU /g. *V. vulnificus* was effectively detected on TSA-2N or mCC10 plates, in which 86% or 84% of the inocula were recovered, respectively (Figure 2C and D). Linearity of regression ($R^2 = 0.75$ on TSA-2N and $R^2 = 0.81$ on mCC10) was observed over the range of 1log–5log CFU/g (Figure 2C and D). In both cases, by increasing the number of selective plates, the LOD could be lowered to 20 CFU/mL (five plates) from 33 CFU/mL (three plates) using DP estimation.

In total, 531 mollusk samples (oysters, clams, and mussels) from Canadian harvest sites on the Atlantic and Pacific coasts were tested seasonally (May–October) between 2002 and 2013, yielding several *Vibrio* spp., particularly the targeted species *V. parahaemolyticus* and *V. vulnificus*, which were confirmed and characterized at the species level (data to be published elsewhere to describe *Vibrio* surveillance analysis and trend) by using this method.

Conclusions

The protocol that we developed using PCR and standard biochemical analyses proved to be sensitive and specific for species confirmation of *V. parahaemolyticus* and *V. vulnificus*. It is a reliable and cost-effective approach for monitoring the presence of potentially pathogenic *Vibrio* spp. in seafood. As such, it will help to gather data to support risk assessments and potentially serve as a foundation for future guidelines and/or food safety regulations. The PCR procedures that were developed have the added flexibility of accommodating other primer pairs amplifying species-specific genes in addition to what has been described here. Although this is a single-laboratory validation procedure, testing by multiple researchers at various times compensated for the within-laboratory and user-method biases. In addition, the approach is transferable to routine microbiological laboratories for simultaneously screening for the presence of halophilic and clinically significant *Vibrio* spp. in seafood samples. This basic protocol is open-ended, with scope for expansion, and is versatile and robust enough to withstand acceptable differences in equipment models between laboratories. Although *V. parahaemolyticus* and *V. vulnificus* were the targeted species, the method was also able to detect other *Vibrio* spp., including *V. alginolyticus* (high prevalence in Canada), *V. cholerae* (detected in recent times), and *V. fluvialis*, demonstrating its versatility. This protocol can be further used in an interlaboratory study for conventional (full) validation and widespread acceptance.

Acknowledgments

We thank Leanne DeWinter (peer review), Nathalie Corneau (peer review), Jennifer Ronholm (Visiting Fellow, manuscript preparation and review), and Neda Nasheri (peer review) of our division (Health Canada, Bureau of Microbial Hazards, Ottawa, ON, Canada) for offering their expertise and helpful comments. We are also thankful for technical assistance from Xiaodong Wan (technical assistance), Rachel Adm (technical help, retired), Sana Sultan (student, University of Ottawa, technical help 2004–05), Laura Petrunka (coop student, 2014, University of Ottawa), Fiona Lau (Honours student, University of Ottawa, 2015), Kassandra Ma (coop student, University of Waterloo, 2016), Mercedes DiBernardo (coop student, University of Waterloo, 2016), and Rebecca Rutley (student, University of Guelph, 2014; technical assistance) at various times for verification and applicability of this method.

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