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Research Note

Evaluation of the Compact Dry VP Method for Screening Raw Seafood for Total *Vibrio parahaemolyticus*

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ABSTRACT

Compact Dry VP (CDVP) is a ready-to-use method for enumerating *Vibrio parahaemolyticus* in food. The presterilized plates contain a culture medium comprising peptone, NaCl, bile salts, antibiotics, chromogenic substrates, and polysaccharide gum as a cold water–soluble gelling. After diluting raw seafood samples in a phosphate-buffered saline solution, a 1-ml aliquot was inoculated onto the center of the plate and allowed to diffuse by capillary action. Blue-green colonies forming on the plates were counted after 18 to 20 h of incubation at 35°C. A total of 85 *V. parahaemolyticus* strains (62 *tdh*$^+$ strains and 23 *tdh*$^-$ strains) were studied for inclusivity, 81 (95.3 %) of which produced blue-green colonies. When 97 strains (14 strains of *Vibrio* spp., 33 strains of coliform bacteria, and 50 strains of noncoliform bacteria) were assessed for exclusivity, 10 strains of *Vibrio* spp. produced non–blue-green colonies, and 87 strains failed to grow. The CDVP and U.S. Food and Drug Administration *Bacteriological Analytical Manual* (FDA-BAM) methods were compared with the use of four different types of raw seafood that were inoculated with four different *V. parahaemolyticus* strains. For raw tuna and oysters, the FDA-BAM colony lift method was used, whereas the FDA-BAM most-probable-number method was used for salmon and scallop. The linear correlation coefficients between the CDVP and FDA-BAM methods were 0.99 for fresh raw tuna, 0.95 for fresh raw oysters, 0.95 for frozen raw salmon, and 0.95 for frozen raw scallops. These results suggest that the CDVP method is useful for screening raw seafood for *V. parahaemolyticus.*

Vibrio parahaemolyticus is a gram-negative halophilic bacterium that has been isolated from various parts of the water column, sediment, zooplankton, shellfish, and fish. It has been found within shellfish as well as codfish, sardine, mackerel, and flounder *(15)* and in environmental samples in Japan *(1). V. parahaemolyticus* is a major foodborne pathogen that causes health problems worldwide *(2, 3, 5–7, 13, 20)* characterized by diarrhea, vomiting, and abdominal cramps through the consumption of contaminated raw or undercooked shellfish. This foodborne pathogen, first isolated in Japan from shellfish-implicated food poisoning *(12),* is now a major cause of food poisoning associated with the ingestion of raw fish as sashimi and sushi *(9).* Hence, it is important to prevent *V. parahaemolyticus* contamination of seafood not only in Japan but also in other parts of Asia because consuming raw fish dishes is a long-standing tradition *(8).*

Efficient analytical methods for the detection of *V. parahaemolyticus* in foods and the environment are needed to establish effective control measures that will reduce the risk of *V. parahaemolyticus* foodborne illness. One common method for detecting *V. parahaemolyticus* in foods is the most-probable-number (MPN) procedure with the use of selective enrichment in alkaline peptone water or salt polymyxin broth and thiosulfate–citrate–bile salts–sucrose (TCBS) agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) according to the U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) *(24).* However, this method (BAM-MPN) is cumbersome and requires at least 3 days to obtain a result. *V. parahaemolyticus* colonies on TCBS agar are difficult to distinguish visually from some strains of *Vibrio mimicus* and *Vibrio vulnificus,* and they can be covered by sucrose-fermenting bacteria that form yellow colonies. The BAM colony lift method (BAM-CL) specifically detects virulence strains that produce thermostable direct hemolysin. Pathogenic strains of *V. parahaemolyticus* are less common in foods *(22, 23).* The Compact Dry VP (CDVP; Nissui Pharmaceutical Co.) method uses the Compact Dry system (Nissui Pharmaceutical Co.) *(21)* containing a culture medium (peptone, NaCl, bile salts, antibiotics, and chromogenic substrates) and a gelling agent such as polysaccharide gum to screen raw seafood simply and rapidly for total *V. parahaemolyticus.* After inoculating 1 ml of sample and incubating at 35°C for 18 to 20 h, *V. parahaemolyticus* can grow as a blue-green colony on the surface or subsurface of the plate. The CDVP method also reduces time and labor costs because the CDVP plates are presterilized by the electron beam and ready to use. The culture medium includes 2% NaCl, inhibitory substances

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(bile salts and antibiotics) for *Enterobacteriaceae* and gram-positive bacteria, and two chromogenic substrates for β-glucosidase and β-galactosidase, which differentiate *V. parahaemolyticus* from other bacteria, unlike traditional growth media such as TCBS agar, in which differentiation is based on sugar fermentation. In this study, the CDVP and FDA-BAM methods were compared. The BAM-MPN method was used for processed seafood because of injured cells, whereas the BAM-CL method was used for molluscan shellfish and fresh raw fish, which were less likely to contain injured bacteria. Because screening followed by extensive biochemical characterization of large numbers of isolates is laborious and time-consuming as well as expensive, the CDVP, a multitest that allows for presumptive identification, offers several advantages. The aim of this study was to evaluate the CDVP method as a means to screen raw seafood total *V. parahaemolyticus.*

MATERIALS AND METHODS

Inclusivity and exclusivity studies. Inclusivity studies were carried out with 85 *V. parahaemolyticus* strains, whereas exclusivity studies were conducted with 14 non–*V. parahaemolyticus* strains, 33 strains of coliform bacteria, and 50 strains of noncoliform bacteria. Strains of *Vibrio* were cultured in tryptic soy broth (TSB; Difco, Becton Dickinson, Detroit, MI) supplemented with 1.5% NaCl (1.5% NaCl-TSB) at 35°C for 20 h. Coliform and noncoliform bacteria were cultured in TSB at 35°C for 24 h. Each bacterial culture was serially diluted in phosphate-buffered saline solution (PBS; Nissui Pharmaceutical Co.) to a population of 25 to 250 CFU/ml, after which 1 ml of this suspension was inoculated onto CDVP and incubated at 35°C for 18 to 20 h.

Method comparison study. The CDVP method was compared with BAM-MPN with the use of frozen raw seafood (salmon and scallops) and to the BAM-CL method with fresh raw seafood (tuna and oysters). All seafood samples were purchased from retail stores in Japan and were confirmed negative for *V. parahaemolyticus* by the FDA-BAM method before inoculation. On the basis of findings from 11 previous cases of *V. parahaemolyticus* foodborne illness in Japan *(14),* contamination levels were as follows: three cases were less than 100 CFU/g, six cases were 100 to 1,000 CFU/g, and 2 cases were more than 1,000 CFU/g. Therefore, the seafood samples (500 g each) were inoculated at the following levels—low: 1 to 2 log CFU/g, medium: 2 to 3 log CFU/g, and high: 3 to 4 log CFU/g. Four strains of *V. parahaemolyticus* were used separately for inoculation: RIMD 2212197 for tuna and RIMD 2210371 for salmon (both from Research Institute for Microbial Diseases, Osaka University, Japan), ATCC 27969 for oysters (American Type Culture Collection, Manassas, VA), and BML 58-9310 for scallops (BioMedical Laboratories, Inc., Saitama, Japan). These four *V. parahaemolyticus* were all of Japanese origin and contained the thermostable direct hemolysin (*tdh*) gene. Five 50-g samples of frozen raw salmon, frozen raw scallops, and fresh raw tuna at each inoculation level were added to 450 ml of PBS (Nissui Pharmaceutical Co.) and blended for 1 min at 8,000 rpm (CELL MASTER CM-100, AS ONE Co., Osaka, Japan). The meat of 12 oysters was pooled into a sterile blender jar, diluted 1:1 in PBS, blended for 90 s at 14,000 rpm, then diluted 1:5 in PBS to obtain a final 1:10 dilution from which additional 10-fold dilutions were prepared as needed.

CDVP. One milliliter of each dilution was inoculated onto a CDVP plate. After 18 to 20 h of incubation at 35°C, the bluish green colonies (Fig. 1) were confirmed as *V. parahaemolyticus* according to the FDA-BAM method.

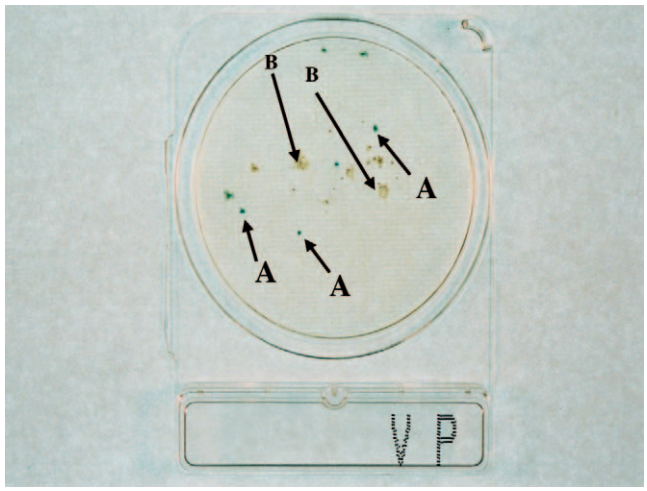

FIGURE 1. *CDVP showing blue-green colonies (A) of* V. parahaemolyticus *ATCC 27969 and light magenta colonies (B) of* V. vulnificus *ATCC 29307 after 20 h of incubation at 35°C.*

BAM-CL. Oysters and tuna homogenates were surface-plated on plates of T_1N_3 that contained 1% (wt/vol) tryptone, 3% (wt/vol) NaCl, and 2% (wt/vol) agar (pH 7.4). After 18 to 24 h of incubation at 35°C, the T_1N_3 plates were overlaid with filters (Whatman 541, Whatman Japan K.K., Tokyo, Japan) for 10 min. Filter preparation, hybridization, and colorimetric detection were as described by Wright et al. *(25)* for *V. vulnificus,* except that hybridization was done at 54°C. Filters with the colony side up were transferred to glass petri dish lid containing 1 ml of lysis solution and then microwaved for 20 s per filter. After drying, the filters were neutralized with ammonium acetate (4 ml per filter) for 5 min at room temperature, rinsed twice in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) buffer (10 ml per filter) and then incubated in proteinase K solution for 30 min at 42°C with shaking (50 rpm) to destroy naturally occurring alkaline phosphatase and digest bacterial protein. The filters were then rinsed three times in 1× SSC for 10 min at room temperature with shaking (50 rpm) and then presoaked in hybridization buffer for 30 min at 54°C with shaking (50 rpm). The hybridization buffer was poured off, and 10 ml of fresh prewarmed buffer and filter was added. The thermolabile hemolysin (*tlh*) gene *(18)* was detected with an alkaline phosphatase (AP)–labeled probe (DNA Technology A/S, Risskov, Denmark). The sequence for the *tlh* probe was 5′-XAA AGC GGA TTA TGC AGA AGC ACT G-3′, with X denoting alkaline phosphatase–conjugated 5′ amine-C6. The *tdh* gene *(19)* was detected with the AP-labeled probe (DNA Technology A/S). The sequence for the *tdh* probe was 5′-XGG TTC TAT TCC AAG TAA AAT GTA TTT G-3′, with X denoting alkaline phosphatase–conjugated 5′ amine-C6. Duplicate filters were used for these probes. Each probe (final concentration 0.5 pmol/ml) was incubated with the filter for 1 h at 54°C with shaking. After 10-min rinses in 1× SSC–1% sodium dodecyl sulfate (for *tlh*) and 3× SSC–1% sodium dodecyl sulfate (for *tdh*) (10 ml per filter) at 54°C with shaking, the filters were developed in 20 ml of nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (Roche, Indianapolis, IN). After hybridization, the filters were covered to omit light, incubated with shaking at room temperature, and subjected to three 10-min rinses to stop development. Finally, the purple or brown spots were counted for enumeration of *V. parahaemolyticus.*

TABLE 1. *Exclusivity study for the CDVP method*

Name of organism	No. of tested strains	No. of growth strains[a]
Vibrio spp.		
V. aestuarianus	1	0
V. alginolyticus	2	2 (C)
V. cholerae	2	2 (M)
V. fluvialis	2	2 (C)
V. hollisae	1	0
V. mimicus	1	1 (LM)
V. orientalis	1	0
V. penaeicida	1	1 (LC)
V. vulnificus	2	2 (LM)
Photobacterium damselae[b]	1	0
Subtotal	14	10
Coliform bacteria		
Enterobacter spp.		
E. aerogenes	1	0
E. agglomerans	1	0
E. Americana	1	0
E. amnigenus	1	0
E. asburiae	1	0
E. cancerogenus	1	0
E. cloacae	1	0
E. gergoviae	1	0
E. sakazakii	1	0
Escherichia spp.		
E. blattae	1	0
E. fergusonii	2	0
E. coli	11	0
E. coli O111	2	0
E. coli O157:H7	2	0
E. hermanii	1	0
Klebsiella spp.		
K. oxytoca	1	0
K. ozaenae	1	0
K. pneumoniae	2	0
K. terrigena	1	0
Subtotal	33	0
Noncoliform bacteria		
Achromobacter xylosoxidans		
subsp. *denitrificans*	1	0
subsp. *xylosoxidans*	1	0
Acinetobacter baumanii	1	0
A. calcoaceticus	2	0
Aeromonas hydrophila	1	0
Alcaligenes faecalis	3	0
Bacillus cereus	2	0
Edwardsiella tarda	3	0
Micrococcus luteus	1	0
M. lylae	1	0
Moraxella nonliquefaciens	1	0
M. ovis	1	0
Proteus mirabilis	1	0
P. vulgaris	2	0
Providencia alcalifaciens	2	0
Pseudomonas spp.		
P. aeruginosa	1	0
P. alcaligenes	1	0
P. diminuta	2	0
P. mendocina	1	0
P. pseudoalcaligenes	1	0
P. putida	1	0
P. stutzeri	1	0
P. vesicularis	1	0
Salmonella Choleraesuis	2	0
Salmonella Typhimurium	*3*	*0*
Shigella boydii	2	0
S. flexineri	2	0
Staphylococcus aureus	3	0
Streptococcus spp.		
S. agalactiae	1	0
S. bovis	1	0
S. canis	1	0
S. equines	1	0
S. salivarius	1	0
S. uberis	1	0
Subtotal	50	0
Total	97	0

[a] Parentheses indicate colony color: C, cream; LC, light cream; LM, light magenta; M, magenta.

[b] *Photobacterium damselae* is a junior objective synonym of *Vibrio damselae*.

BAM-MPN. A homogenate in alkaline peptone water supplemented with 2% (wt/vol) NaCl was used to prepare a three-tube MPN dilution series starting with 0.1-g portions and continuing with 10-fold dilutions to a 10^{-3} dilution. After overnight enrichment at 35°C, all tubes were streaked to TCBS agar. After overnight incubation at 35°C, typical colonies (round, 2 to 3 mm in diameter, smooth, opaque, and green or blue-green) were selected and biochemically confirmed as *V. parahaemolyticus* by traditional biochemical tests (oxidase, growth in 3 to 10% [wt/vol] NaCl, growth at 42°C, and sensitivity to 10 μg of O/129) and API 20E (bioMérieux Japan Ltd., Tokyo, Japan). These confirmed isolates were then used in determining the MPN from the three-tube MPN tables.

Statistical analysis. Results were calculated as log *V. parahaemolyticus* CFU per g of food. All statistical analyses were carried out with the Microsoft Excel 2000 statistics package and performed at a significance level of 0.05. A one-way analysis of variance (ANOVA) was performed to determine differences between the CDVP and FDA-BAM methods with the slopes, intercepts, and squares of the linear correlation coefficients (r^2) then calculated.

RESULTS AND DISCUSSION

A total of 85 *V. parahaemolyticus* strains were examined by the CDVP method in the inclusivity study. Eighty-one strains (95.3%) produced blue-green colonies, with four white colonies negative for β-glucosidase and β-galactosidase. However, additional *V. parahaemolyticus* strains from various areas in the world need to be assessed to determine specificity. Results from the exclusivity study are shown in Table 1. Four of 14 non–*V. parahaemolyticus Vibrio* strains failed to grow, with 10 others producing magenta or cream colonies. None of the 83 non-*Vibrio* strains (33 coliform

TABLE 2. *Results of enumeration with the CDVP and FDA-BAM methods in artificially contaminated raw seafood*[a]

Level of artificial contamination	Mean *V. parahaemolyticus* (log CFU/g)				Mean *V. parahaemolyticus* (log MPN/g)			
	Fresh raw oysters		Fresh raw tuna		Frozen raw salmon		Frozen raw scallops	
	CDVP	BAM-CL	CDVP	BAM-CL	CDVP	BAM-MPN	CDVP	BAM-MPN
Low (1–2 log CFU/g)								
Mean	1.60	1.62	2.05	2.12	1.52	1.46	1.49	1.47
SD	0.28	0.08	0.15	0.16	0.35	0.23	0.14	0.15
P	0.89		0.47		0.77		0.85	
Intermediate (2–3 log CFU/g)								
Mean	2.51	2.65	3.07	3.00	2.45	2.58	2.33	2.47
SD	0.17	0.29	0.08	0.13	0.36	0.29	0.19	0.15
P	0.38		0.39		0.55		0.24	
High (3–4 log CFU/g)								
Mean	3.60	3.76	4.42	4.43	3.57	3.73	3.60	3.71
SD	0.08	0.15	0.18	0.13	0.21	0.13	0.07	0.26
P	0.07		0.93		0.37		0.19	

[a] Oysters were inoculated with *V. parahaemolyticus* ATCC 27969, tuna was inoculated with *V. parahaemolyticus* RIMD 2212197, salmon was inoculated with *V. parahaemolyticus* RIMD 2210371, and scallops were inoculated with *V. parahaemolyticus* BML 58-9310.

and 50 noncoliform bacteria) grew. Table 2 shows the mean *V. parahaemolyticus* (log CFU per gram) for the CDVP and FDA-BAM methods at each inoculation level. No significant difference was seen between the CDVP and FDA-BAM methods by one-way ANOVA ($P > 0.05$).

The r^2, slopes, intercepts, and 95% confidence limits for the CDVP and FDA-BAM methods are presented in Table 3. The r^2 values between the CDVP and BAM-CL methods were 0.99 for fresh raw tuna and 0.95 for fresh raw oysters. The r^2 values between the CDVP and BAM-MPN methods were 0.95 for frozen raw salmon and 0.95 for frozen raw scallops. For all comparisons, the slope and intercept values, as determined by linear regression analysis, were close to 1.00 and 0.00, respectively. The CDVP yielded consistent results with each inoculum level by comparison testing. The limit of detection for the CDVP method was 10 CFU/g.

TABLE 3. *Relationship between the CDVP and FDA-BAM methods for enumerating* V. parahaemolyticus *in raw seafood*[a]

Parameter	Fresh raw oysters	Fresh raw tuna	Frozen raw salmon	Frozen raw scallops
No. of samples[b]	15	15	15	15
Correlation coefficient	0.95	0.99	0.95	0.95
Slope	1.04	0.97	1.05	1.04
Intercept	0.01	0.10	−0.04	−0.02
95% confidence limits	±0.47	±0.18	±0.49	±0.46

[a] Oysters were inoculated with *V. parahaemolyticus* ATCC 27969, tuna was inoculated with *V. parahaemolyticus* RIMD 2212197, salmon was inoculated with *V. parahaemolyticus* RIMD 2210371, and scallops were inoculated with *V. parahaemolyticus* BML 58-9310.

[b] Five samples at three contamination levels were analyzed for each food type.

In Japan the acceptable level for total *V. parahaemolyticus* in fish fillets and shocked shell fish to be consumed raw is below 100 CFU/g. Some molecular methods, such as PCR *(4, 11, 16)*, real-time PCR *(10)*, and the transcription–reverse transcription concerted method *(17)* have been developed for rapid detection of *V. parahaemolyticus* and the *tdh, tlh,* or both genes. These molecular methods have become vital epidemiological tools for detecting and characterizing bacteria associated with foodborne outbreaks *(10)*. However, execution of these molecular methods as an independent hygiene control is difficult during transport, processing, and storage because of the equipment and skills needed to perform the test. Although the CDVP method has shortcomings similar to culture-based methods, it is less costly and far easier to set up and perform compared with the BAM-MPN and BAM-CL methods. Consequently, the CDVP method appears useful in screening raw seafood for total *V. parahaemolyticus* in the field.

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