P4-04 Novel Method for Screening Raw Seafood for *Vibrio parahaemolyticus*

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【INTRODUCTION】

Vibrio parahaemolyticus is a major food-borne pathogen that causes worldwide health problems (*1*) characterized by diarrhea, vomiting, and abdominal cramps through the consumption of contaminated raw or undercooked shellfish since *V. parahaemolyticus* was first isolated from shellfish implicated food poisoning in Japan (*2*). Prevention of *V. parahaemolyticus* contamination of seafood is an important public health concern.

One of the common methods for detection of *V. parahaemolyticus* in foods is the most probable number (MPN) procedure using selective enrichment with alkaline peptone water or salt polymyxin broth and thiosulfate-citrate-bile salts-sucrose agar (TCBS) according to the U. S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) method (*8*). This method (BAM-MPN) is cumbersome and requires at least 3 days to get a result. *V. parahaemolyticus* colonies on TCBS agar are difficult to distinguish visually from other some strains of *Vibrio mimicus* and *Vibrio vulnificus*, and they can be covered by sucrose-fermenting bacteria forming yellow color colonies. The BAM colony lift method (BAM-CL) is specific detection method for detection of the thermostable direct hemolysin (*tdh*) gene of virulence strains. Pathogenic *V. parahaemolyticus* has not been as frequently detected in foods as it has been in clinical specimens (*7*). The Compact Dry VP™ (CDVP) method uses the Compact Dry™ system (*6*) with the culture medium (peptone, sodium chloride, bile salts, antibiotics, and chromogenic substrates) and a gelling agent, and has been developed to simply and rapidly screen raw seafood for total *V. parahaemolyticus*. The CDVP™ plates are sterilized by the electron beam and ready to use for cultivation of total *V. parahaemolyticus*. The CDVP™ method becomes a reduction in the working hour and the labor cost because of not necessary for preparing media. The CDVP™ method was compared with BAM. BAM-MPN was used for the processed product because of injured cells, and BAM-CL was used for molluscan shellfish and fresh raw fish because it was thought that it did not contain the injured bacteria. The aim of this study was to evaluate the CDVP™ to adapt to screening raw seafood for total *V. parahaemolyticus*.

【MATERIALS AND MRTHODS】

Method comparison study.

The CDVP™ method was compared with BAM-MPN for frozen raw seafood (salmon and scallops) and with BAM-CL for fresh raw seafood (tuna and oysters). BAM-MPN was used for the processed product because of injured cells, and BAM-CL was used for fresh raw fish because it was thought that it did not contain the injured bacteria. Every sample was purchased from retail stores in Japan. All seafood was confirmed negative for *V. parahaemolyticus* by the FDA-BAM method before artificial contamination. According to investigation of 11 *V. parahaemolyticus* foodborne outbreak cases in Japan (*3*), food contamination levels were as follows: 3 cases were < 100 CFU/g, 6 cases were 100 - 1000 CFU/g，and 2 cases were > 1000 CFU/g. Therefore, artificially contaminated seafood samples were selected and prepared at three contamination levels (low: log 1-2 CFU/g, intermedium: log 2-3 CFU/g, and high: log 3-4 CFU/g). Each contamination level was approximately 1 log higher than the previous. Four strains of *V. parahaemolyticus,* RIMD 2212197 for tuna and RIMD 2210371 for salmon from the Research Institute for Microbial Diseases (Osaka University, Japan), ATCC 27969 for oysters from American Type Culture Collection (Va., USA), and BML 58-9310 for scallops from Bio Medical Laboratories, Inc. (Saitama, Japan) were used separately for each inoculated food type. Five 50g frozen raw salmon, frozen raw scallops, and fresh raw tuna each in 450 ml phosphate buffered saline solution (PBS; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) was blended for 1 min at 8000 rpm. Twelve oyster meats were pooled into a sterile blender jar. An equal volume of PBS was added, and samples were blended for 90 sec at 14000 rpm.

CDVP™. One ml of each dilution was inoculated onto CDVP™ (Nissui Pharma.) (Fig. 1) which consists of an unwoven cloth with selective and different culture medium for *V. parahaemolyticus*, a cold water-soluble gelling agent, and a unique plastic dish (Fig.1). The plates were incubated at 35°C for 18-20 h and the blue colonies grown on the surface and subsurface were counted as number of *V. parahaemolyticus*.

BAM-CL. Oysters were spread 0.2 g of the 10^{-1} dilution and 0.1 ml of the 10^{-2}, and 10^{-3} dilutions onto T_1N_3 (1% tryptone, 3% NaCl, 2% agar, pH 7.4) plates. Tuna was spread 0.1 ml of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions onto T_1N_3 plates. After 18-24 h 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 done described by Wright et al. (*9*) for *V. vulnificus*, except that hybridization was done at 54°C. The hybridization procedures were carried out by FDA-BAM. The thermolabile hemolysin gene (*tlh*) (*4*) was detected by using by the alkaline phosphatase (AP) - labeled probe (DNA Technology A/S, Denmark). The *tdh* gene (*5*) was detected by using by the AP-labeled probe (DNA Technology A/S, Denmark). The filters were developed with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt (NBT/BCIP; Roche; Ind., USA). Twenty ml of NBT/BCIP solution was added in petri dish. The purple or brown spots were counted for the enumeration of *V. parahaemolyticus*.

BAM-MPN. Alkaline peptone water supplemented with 2% NaCl was used in determination of MPN counts. The homogenate was used to prepare a 3-tube-MPN dilution series starting with 0.1-gram portions and continuing with 10-fold dilutions to a 10^{-3} dilution. After overnight enrichment at 35°C, all tubes were streaked to thiosulfate-citrate-bile salts-sucrose (TCBS; Nissui Pharma.) agar. After overnight incubation at 35°C, typical colonies (round, 2 to 3 mm in diameter, smooth, opaque, and green or blue-green colonies) were isolated from TCBS plates and biochemically confirmed as *V. parahaemolyticus* using traditional biochemical tests (oxidase, growth in 3-10 % NaCl, growth at 42°C, and sensitivity to 10 μg O/129) and API 20E (bioMerieux Japan, Ltd., Tokyo, Japan). After the colonies were finally identified as *V. parahaemolyticus* these were referred back to the original dilutions in the enrichment broth and then 3-tube-MPN tables were used for final enumeration of the organism.

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

【RESULTS AND DISCUSSION】

According to the results of the enumeration with the CDVP™ and FDA-BAM methods for *V. parahaemolyticus* for each artificially contamination level, there was no significant difference between the CDVP™ and FDA-BAM methods by one-way ANOVA (P > 0.05). The difference was not observed in the results that each strain was being studied for one strain for one type of seafood. The r^2 between the CDVP™ and BAM-CL methods were 0.99 for fresh raw tuna (Fig. 2) and 0.95 for fresh raw oysters (Fig. 3). The r^2 between the CDVP™ and BAM-MPN methods were 0.95 for frozen raw salmon (Fig. 4) and 0.95 for frozen raw scallops (Fig. 5). 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 detection limit of CDVP™ method is 10 CFU/g. The CDVP™ method has similar shortcomings as other culture-based methods. The CDVP™ method is reducing incubation space and relatively cheaper (1plate = about 1 US$) than BAM-MPN or BAM-CL methods. The CDVP™ method is also easier to set up without any preparation media than BAM-MPN and is simpler to use without technical expertise than BAM-CL. The shelf life of the CDVP™ is one and half year at room temperature after manufacturing. The CDVP™ would be useful for screening raw seafood for *V. parahaemolyticus* on the field and provide food safety for raw seafood.

【ACKNOWLEDGMENTS】

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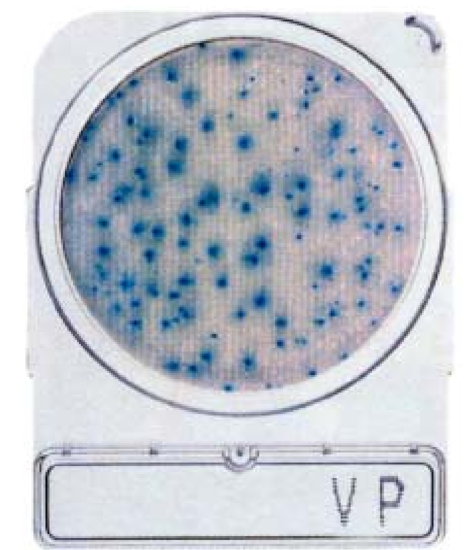

Figure 1. Colony formation of *V. parahaemolyticus* on Compact Dry VPTM
V. parahaemolyticus ATCC 27969 was incubated at 35C for 20 h.

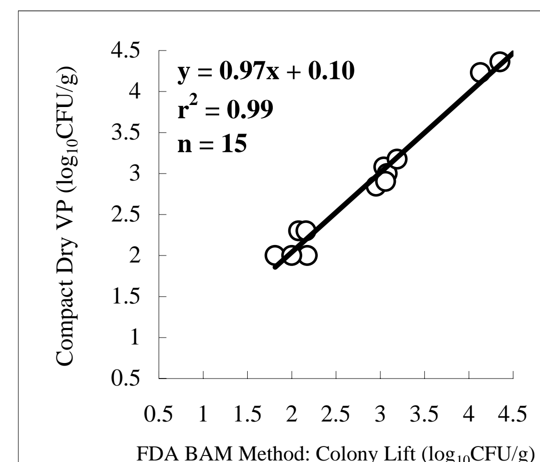

Figure2. Correlation between Compact Dry VP and FDA BAM Method: Colony Lift for *V. parahaemolyticus* in fresh raw tuna.

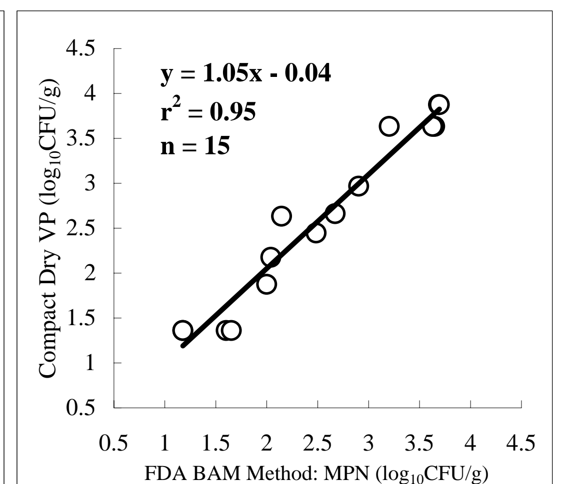

Figure 4. Correlation between Comapct Dry VP and FDA BAM Method: MPN for *V. parahaemolyticus* in frozen raw salmon.

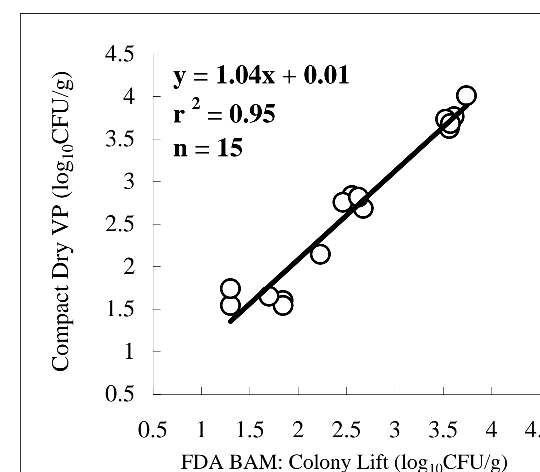

Figure 3. Correlatioo between Comapct Dry VP and FDA BAM Method: Colony Lift for *V. parahaemolyticus* in fresh raw oysters

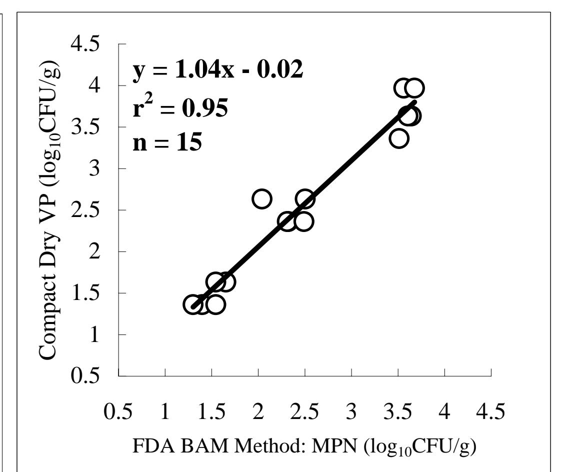

Figure 5. Correlation between Compact Dry VP and FDA BAM Method: MPN for *V. parahaemolyticus* in frozen raw scallops.