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Full Length Research Paper

Evaluation of a new chromogenic selective medium for isolation and enumeration of *Vibrio parahaemolyticus*

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The X-VP agar, chromogenic selective medium for *Vibrio parahaemolyticus*, was evaluated for inclusivity and exclusivity by using 169 strains including 55 *V. parahaemolyticus* and compared with Thiosulfate Citrate Bile salt Sucrose (TCBS) and CHROMagar Vibrio media as conventional methods using *V. parahaemolyticus* inoculated seafood samples. Our results suggested the X-VP agar was useful for isolation and enumeration of *V. parahaemolyticus*.

Key words: Chromogenic selective agar medium, seafood, *Vibrio parahaemolyticus*, X-VP agar.

INTRODUCTION

Vibrio parahaemolyticus is known as a gram-negative halophilic marine bacterium that is found widely in seawater, brackish water, sediment, fish and shellfish (Chakraborty et al., 1997; Oliver and Kaper, 1997; Su and Liu, 2007). *V. parahaemolyticus* causes diarrhea and abdominal pains through the consumption of contaminated seafood (Fujino et al., 1953; Sakazaki et al., 1968). *V. parahaemolyticus* has been the major pathogen for a long time in Japan because of habitual for eating raw fish and shellfish as sashimi and sushi (Alam et al., 2002; Hara-Kudo et al., 2003; Otomo, 2000).

Since 2001, the acceptable level for fresh seafood, frozen seafood and raw oyster has been established as below 100 cfu/g in Japan. Since then, even though food poisoning by *V. parahaemolyticus* has been decreasing, *V. parahaemolyticus* food poisonings have still been occurred several dozen times a year in Japan (Ministry of Health Labour and Welfare, 2009).

The Thiosulfate Citrate Bile salt Sucrose (TCBS) agar is used for detection of *V. parahaemolyticus* in worldwide (Donovan and van Netten, 1995; Kobayashi et al., 1963). However, in case that many acid-producing bacteria from sucrose such as *V. alginolyticus* grew on TCBS, colonies of *V. parahaemolyticus* tend to be masked by color change. Meanwhile, the CHROMagar™ Vibrio (CV; CHROMagar Microbiology, Paris, France) has been developed as choromogenic selective medium for *V. parahaemolyticus*. Hara-Kudo et al. (2001) reported CV enables presumptive differentiation for *V. parahaemolyticus.* However, it is not only hard to obtain at any time in Japan but also has high cost because it is imported product. In these backgrounds, the X-VP agar (X-VP; Nissui Pharmaceutical Company, Limited, Tokyo, Japan) has been developed as cost-effective domestic chromogenic selective agar consisting of nutrients, NaCl, antibiotics and two chromogenic substrates for ß-glucosidase and ß-galactosidase which differentiate *V. parahaemolyticus* from other bacteria. The aim of this study was to evaluate the X-VP for its performance in isolation and enumeration of *V. parahaemolyticus*.

MATERIALS AND METHODS

X-VP agar

X-VP agar was developed primarily for selective for Vibrionaceae. The medium contains the following ingredients per liter: peptone (Difco, Becton Dickinson, Detroit, MI, USA), 10.0 g; yeast extract (Difco), 5.0 g; NaCl, 20.0 g; sucrose, 30.0 g; ox bile (American

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Laboratories, Inc., Omaha, NE, USA), 0.25 g; sodium cholate, 3.0 g; sodium citrate, 10.0 g; sodium thiosulfate, 6.4 g; sodium pyruvate, 5.0 g; vancomycin hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.02 g; 5-Bromo-4-Chloro-3-Indolyl-Beta-D-Glucopyranoside (Biosynth AG, Staad, Switzerland), 0.15 g; 5-Bromo-6-chloro-3-indoxyl-beta-D-galactopyranoside (Biosynth AG), 0.10 g; and agar, 12.5 g (pH 8.8).

Samples

For comparison studies, 12 fresh shrimps, 12 clams, 12 filleted fishes, and 12 oysters were purchased from retail stores in Japan.

Inclusivity and exclusivity studies

Ninety-five of *Vibrio* strains including 55 *V. parahaemolyticus* strains were inoculated for the inclusivity study. For the exclusivity study, 40 gram-negative strains other than *Vibrio* strains, 31 gram-positive strains and 3 yeasts were inoculated. *Vibrio* strains were cultured in Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Detroit, MI, USA) with 2% NaCl at 35°C for 24 h. Bacterial strains other than *Vibrio* strains were cultured in TSB at 35°C for 24 h and yeast strains were cultured in Sabouraud Dextrose Broth (Difco) at 25°C for 72 h. Each culture was streaked onto X-VP, TCBS and CV. The growth and colony color of each tested strain were read after incubation for 24h at 35°C.

Method comparison study

The X-VP was compared with TCBS (Nissui Pharmaceutical Co., Ltd.) and CV using artificially contaminated seafood samples. Fourty-eight *V. parahaemolyticus* negative seafood samples (12 fresh shrimps, 12 clams, 12 filleted fishes, and 12 oysters) were purchased from retail stores in Japan. For the comparison studies, the *V. parahaemolyticus* strains used were: ATCC 17802 and ATCC 33845 (American Type Culture Collection, Manassas, VA.); and NS 7047 isolated from environment. Each 25 g sample was randomly inoculated at the following levels: 2-3 log CFU/g, 3-4 log CFU/g, 4-5 log CFU/g and 5-6 log CFU/g.

After 3 days preservation at 4°C, each artificially contaminated sample was added to a 9-fold volume of PBS (Nissui Pharmaceutical Co., Ltd.) with 2% NaCl and was homogenized for 2 min with a homogenizer (Pro-media SH-001, ELMEX LIMITED, Tokyo, Japan). Subsequently, each homogenized sample was subjected to 10-fold serial dilution by PBS with 2% NaCl. Dual measurements were then carried out for each method. Each 0.1ml samples was spread onto the surface of the X-VP, TCBS and CV with sterilized plastic spreader (Nissui Pharmaceutical Co., Ltd.), respectively. After 24 h at 35°C, the blue colonies on X-VP, green colonies on TCBS and mauve colonies on CV were read as *V. parahaemolyticus*, respectively.

Statistical analysis

Results from method comparison study were converted into log CFU of *V. parahaemolyticus* per gram of each tested food. All statistical analyses were carried out with the Microsoft Excel 2000 at the significance level of P = 0.05. The linear correlation coefficients (*r*), slopes, intercepts between X-VP and TCBS, and X-VP and CV were calculated, respectively. A one-way analysis of variance (ANOVA) was performed to determine differences between X-VP and both methods.

RESULTS AND DISCUSSION

Results from both inclusivity and exclusivity studies are shown in Table 1. Of 55 *V. parahaemolyticus* strains, 53 strains (96.4%) grew as blue colored colony, whereas 2 strains of *V. parahamemolyticus* grew as milk-white colony. Of 40 *Vibrio* strains other than *V. parahaemolyticus*, all of *V. alginolyticus* and *V. fluvialis* grew as milk-white colony, all of *V. cholerae*, *V. mimicus* and *V. vulnificus* grew as magenta colony, and other 6 *Vibrio* strains failed to grow, respectively. A total of 40 gram-negative bacteria, 31 gram-positive bacteria and 3 yeasts failed to grow. In contrast, *Citrobacter freundii*, *C. koseri*, *Klebsiella oxytoca* and *K. ozaenae* were grown on CV.

Figures 1 and 2 show the correlation coefficients (*r*), slopes, intercepts between X-VP and TCBS, and X-VP and CV from method comparison study. The *r* between X-VP and TCBS, and X-VP and CV, were 0.961 and 0.977, respectively. No significant difference was shown between X-VP and both methods by one-way ANOVA (P>0.05), even though TCBS failed to recover *V. parahaemolyticus* from 1 sample.

Although TCBS has a high selectivity for *Vibrio* species strains, TCBS cannot distinguish *V. parahaemolyticus* from other *Vibrio* strains such as *V. vulnificus*. Hence, TCBS needs confirmation tests for precise enumeration. In contrast, X-VP has not only good selectivity for *V. parahaemolyticus*, but also ability for differentiation from other major pathogenic *Vibrio* strains such as *V. vulnificus* which possessing ß-galactosidase as magenta colony. Especially, *V. vulnificus* is known one of bacteria showing critical clinical symptoms (Cerda-Cuellar, 2000, 2001; Chen et al., 2010). Hence, X-VP is useful for screening medium for pathogenic *Vibrio* species. Although 2 strains (3.6%) of 55 *V. parahaemolyticus* grew on X-VP as white colony, this result corresponded to report by Su et al. (2005). Hence, there are *V. parahaemolyticus* lacking ß-glucosidase inconsiderably. However X-VP has a good correlation with TCBS and CV using seafood samples, and no significant difference with these media.

In conclusion, the X-VP is excellent in selectivity and differentiation for *V. parahaemolyticus* and a suitable alternative method for the detection and enumeration of *V. parahaemolyticus* in seafood. The X-VP is supplied promptly and cost-effective in Japan.

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Table 1. Strains tested for growth and color on X-VP.

Name of organism	No. of tested strains			No. of strains grown [b]		
	Standard [a]	Isolate	Total	X-VP	TCBS	CV
***Vibrio* spp.**						
V. aestuarianus	1I	0	1	0	0	0
V. alginolyticus	1I	9	10	10 (W)	9 (Y)	10 (W)
V. cholera	0	6	6	6 (M)	6 (Y)	6 (B)
V. fluvialis	1A, 1J	0	2	2 (W)	2 (Y)	2 (W)
V. hollisae	1J	0	1	0	0	0
V. mimicus	1A	1	2	2 (M)	2 (G)	2 (B)
V. orientalis	1I	0	1	0	0	0
V. parahaemoplyticus	18A, 1I, 9R	27	55	55 53(B), 2(W)	55(G)	55 52(m), 3(W)
V. penaeicida	1I	0	1	0	0	0
V. vulnificus	4A, 7J	3	14	14 (M)	14(G)	14 (B)
Photobacterium damselae [c]	4A, 7J	1	2	0	2 (G)	0
Subtotal	48	47	95	89	90	89
Gram negative bacteria except for *Vibrio* spp.						
Aeromonas hydrophila	1J	0	1	0	0	0
Citrobacter amalonaticus	1A	0	1	0	0	0
C. freundii	1A	0	1	0	0	1 (B) [d]
C. koseri	1A	0	1	0	0	1 (B) [d]
Enterobacter aerogenes	1A	0	1	0	0	0
E. amnigenus	1A	0	1	0	0	0
E. cloacae	1A	1	2	0	0	0
E. intermedius	2A	0	2	0	0	0
E. sakazakii	1A	0	1	0	0	0
Escherichia coli	4A	0	4	0	0	0
E. coli O-157	2A	0	2	0	0	0
E. hermannii	1J	0	1	0	0	0
Hafnia alvei	1A	0	1	0	0	0
Klebsiella oxytoca	1A	0	1	0	0	1 (m) [d]
K. ozaenae	1A	0	1	0	0	1 (m) [d]
K. pneumoniae	1A	1	2	0	0	0
Kluyvera ascorbata	1A	0	1	0	0	0
K. cryocrescens	1A	0	1	0	0	0
Morganella morganii	1A	0	1	0	0	0
Proteus mirabilis	1A	0	1	0	0	0
P. vulgaris	1A	0	1	0	0	0
Pseudomonas aeruginosa	3A	0	3	0	0	0
P. putida	1A	0	1	0	0	0
Rahnella aquatilis	1A	0	1	0	0	0
Salmonella enterica	2A	0	2	0	0	0
Serratia fonticola	1A	0	1	0	0	0
S. liquefaciens	1A	1	2	0	0	0
S. marcescens	2A	0	2	0	0	0
Subtotal	37	3	40	0	0	0
Gram positive bacteria						
Bacillus cereus	1A	0	1	0	0	0
B. licheniformis	1A	0	1	0	0	0
B. subtilis	1A	0	1	0	0	0

Table 1. Contd.

Corynebacterium minutissimum	1A	0	1	0	0	0
C. renale	1A	0	1	0	0	0
C. xerosis	1A	0	1	0	0	0
Enterococcus avium	1A	0	1	0	0	0
E. durans	1A	0	1	0	0	0
E. faecalis	2A	0	2	0	0	0
E. faecium	1A	0	1	0	0	0
Lactobacillus lactis	1A	0	1	0	0	0
Micrococcus luteus	1A	0	1		0	0
Staphylococcus aureus	4A	1	5	0	0	0
S. auricularis	1A	0	1	0	0	0
S. capitis	1A	0	1	0	0	0
S. epidermidis	2A	0	2	0	0	0
S. haemolyticus	1A	0	1	0	0	0
S. hominis	1A	0	1	0	0	0
S. lentus	1A	0	1	0	0	0
S. saprophyticus	1A	0	1	0	0	0
S. sciuri	1A	0	1	0	0	0
S. simulans	1A	0	1	0	0	0
S. warneri	1A	0	1	0	0	0
S. xylosus	1A	0	1	0	0	0
Streptococcus thermophiles	1A	0	1	0	0	0
Subtotal	30	1	31	0	0	0
Yeasts						
Candida albicans	2A	0	2	0	0	0
Saccharomyces cerevisiae	1A	0	1	0	0	0
Subtotal	3	0	3	0	0	0
Total	118	51	169	89	90	93

[a]Standard strains were from A; ATCC (American Type Culture Collection), I; IFO (Institute for Fermentation, Osaka), J; JCM (Japan Collection of Microorganisms) and R; RIMD(Research Institute for Microbial Diseases, Osaka University, Japan); [b] Parentheses indicate colony color: B, blue; M, magenta; W, white; m, mauve; [c]*Photobacterium damselae* is a synonym of Vibrio damsel; [d]Grown at only high density site of streaking.

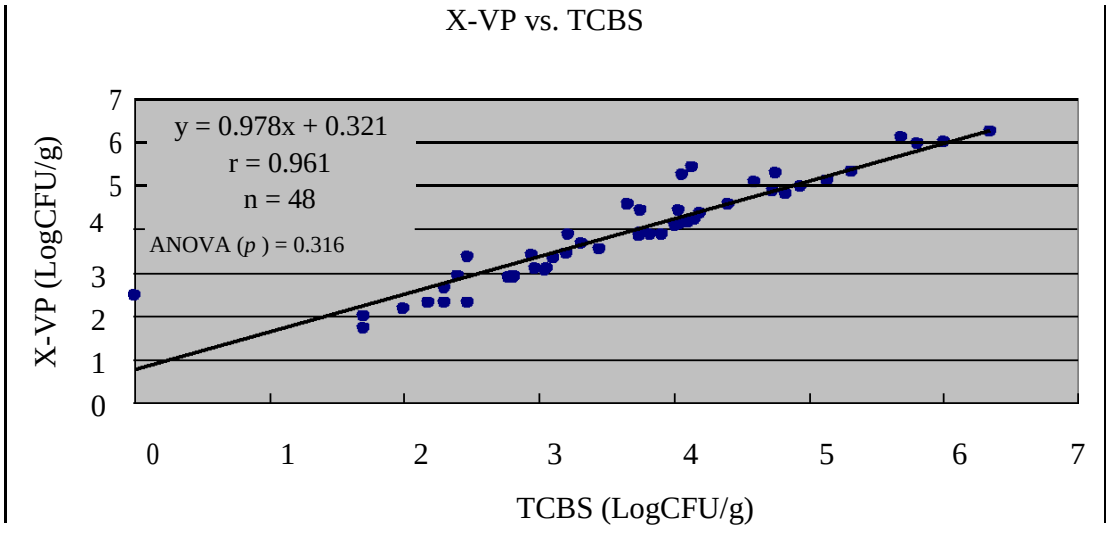

Figure 1. Regression line for data from the X-VP agar method plotted against the TCBS method for determining population of *V. parahaemolyticus* in 48 seafood samples.

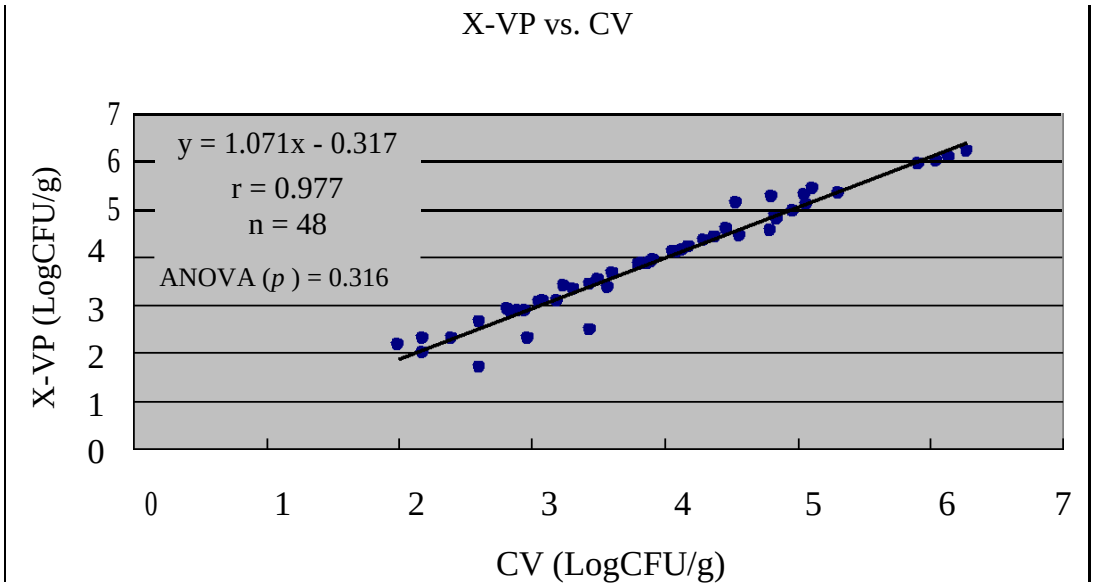

Figure 2. Regression line for data from the X-VP agar method plotted against the CV method for determining population of *V. parahaemolyticus* in 48 seafood samples.

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