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Improved Fluorogenic Assay for Rapid Detection of *Vibrio parahaemolyticus* in Foods

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An improved fluorogenic assay for the rapid detection of *Vibrio parahaemolyticus* was developed. In the improved assay, the enrichment of *V. parahaemolyticus* was carried out in arabinose-glucuronate medium (0.5% arabinose, 0.25% glucuronate, 0.1% polypeptone, 0.1% yeast extract, 0.1% ammonium sulfate, 2% NaCl, 2 μg of polymyxin B sulfate per ml, pH 8.5) at 37°C. After the cultivation, the trypsinlike activity of the bacteria was measured by fluorescence with the fluorogenic substrate benzoyl-L-arginine-7-aminomethylcoumarin. Even in the presence of 3×10^5 cells of *Vibrio alginolyticus*, 20 cells of *V. parahaemolyticus* were clearly detected after a 6-h enrichment cultivation by the assay. Fifty contaminated samples of 14 seafoods were examined for *V. parahaemolyticus* by the fluorogenic assay after enrichment cultivation for 6 or 8 h. The results were then compared with those obtained by the conventional bromothymol blue Teepol agar assay and the most-probable-number method. There was a linear relationship between trypsinlike activity measured by the assay and the number of *V. parahaemolyticus* cells in seafood as determined by the bromothymol blue Teepol agar and most-probable-number methods. Correlation coefficients were 0.95 and 0.93 after a 6-h cultivation and an 8-h cultivation, respectively. The presence of 10 cells of *V. parahaemolyticus* per gram of seafood sample was detected after a 10-h total detection time by the fluorogenic assay.

Most bacterial food poisoning in Japan is caused by *Vibrio parahaemolyticus* in fresh and processed seafood (8). The most common method to detect *V. parahaemolyticus* is a culture procedure using enrichment media and subsequent isolation on selective plating media. Since the conventional detection method requires 2 to 3 days, a more rapid method is required.

We previously developed a rapid and sensitive detection assay in which intracellular trypsinlike activity of *V. parahaemolyticus* was measured by using a fluorogenic substrate (6a). However, on the selective medium, *Vibrio alginolyticus* and *Vibrio harveyi* were able to grow, and a large population of these bacteria may interfere with the detection of *V. parahaemolyticus*.

This paper reports a new selective medium for *V. parahaemolyticus* in which *V. alginolyticus* and *V. harveyi* do not grow and the application of the new medium to the fluorogenic detection of *V. parahaemolyticus* in food.

MATERIALS AND METHODS

Strains. *Escherichia coli* IFO 3301, *Enterobacter aerogenes* IFO 13534, *Pseudomonas fragi* IFO 3458, *Salmonella enteritidis* IFO 3313, *Salmonella typhimurium* IFO 12529, and *Staphylococcus aureus* IFO 3060 were purchased from the Institute for Fermentation, Osaka, Japan. *Bacillus cereus* JCM 2152, *Bacillus subtilis* JCM 1465, *Campylobacter jejuni* JCM 2013, and *Lactobacillus lactis* JCM 1248 were supplied by the Japan Collection of Microorganisms. *V. parahaemolyticus* WP-1, 26-1, 27-2, 33-7, 33-8, 33-10, 39-3, 39-11, and 46-11, *V. alginolyticus* 10-1, 13-2, 14-1, and 14-2, *V. cholerae* FK and non-01 FK, *V. damsela* FK, *V. fluvialis* FK, *V. harveyi* FK, and *V. vulnificus* FK were obtained from the Fukuoka City Institute of Public Health, Fukuoka, Japan. *V. cholerae* NR and non-O1 NR, *V. fluvialis* NR, and *V. furnissii* NR were generously provided by Seiichi Umesako, Nara Prefectural Institute of Public Health, Nara, Japan. *V. anguillarum* Ty 12 and *Aeromonas hydrophila* A 0111003 were kindly provided by Ushio Shimizu, Ocean Research Institute, University of Tokyo, Tokyo, Japan.

Fluorogenic substrate. Benzoyl-L-arginine-7-aminomethylcoumarin (Bz-Arg-7AMC) (Peptide Institute, Inc., Osaka, Japan) was dissolved in dimethyl sulfoxide at a concentration of 6 mM and kept at −20°C.

Media. Polypeptone-meat extract broth (1% polypeptone, 1% meat extract, 2% NaCl, pH 7.0) and arabinose-glucuronate (AG) medium (0.5% arabinose, 0.25% glucuronate, 0.1% polypeptone, 0.1% ammonium sulfate, 0.1% yeast extract, 2% NaCl, 2 μg of polymyxin B sulfate per ml, pH 8.5) were prepared and autoclaved unless otherwise noted. In the case of AG medium, the solutions of arabinose and glucuronate, autoclaved separately, and the solution of polymyxin B sulfate, sterilized with a membrane filter, were added afterward.

Evaluation of selective medium and measurement of intracellular trypsinlike activity. After the bacteria were incubated at 30°C overnight in polypeptone-meat extract broth, the culture broth containing bacterial cells was serially diluted with peptone water (0.1% polypeptone, 2% NaCl, pH 7.0) and used as a bacterial broth. Polypeptone-meat extract broth with 2% NaCl was used to cultivate *Vibrio* species. One milliliter of the bacterial broth was inoculated in 6 ml of AG medium and incubated at 37°C for 6 h. Bacterial cells were harvested by centrifugation (1,400 × *g* for 10 min) of 7 ml of the culture broth. Three milliliters of 50 mM phosphate buffer (pH 7.5) including 0.01 mM Bz-Arg-7AMC and 0.3 ml of 10 mM EDTA was added to the cell pellet, and the buffer was allowed to react with the pellet at 40°C for 1 h. The reaction was stopped by adding 1 ml of 1 M glycine buffer (pH 11.0); then the reaction mixture was centrifuged (1,400 × *g* for 10 min), and the supernatant was used to measure the fluorescence intensity with a fluorospectrometer (FLUORO-READ Model 200, Ajinomoto Co., Inc., Tokyo, Japan). The

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TABLE 1. Trypsinlike activity of various bacteria grown in AG medium[a]

Strain (Kanagawa hemolysin)	Trypsinlike activity
Control	1.0
Vibrio parahaemolyticus WP-1 (+)	42.8
V. parahaemolyticus 27-2 (+)	42.6
V. parahaemolyticus 33-7 (+)	39.1
V. parahaemolyticus 33-10 (+)	46.1
V. parahaemolyticus 26-1 (−)	45.0
V. parahaemolyticus 39-11 (−)	39.0
V. parahaemolyticus 46-11 (−)	42.8
V. alginolyticus 10-1	1.1
V. alginolyticus 13-1	1.4
V. anguillarum Ty 12	1.0
V. cholerae FK	1.0
V. cholerae non-O1 FK	1.0
V. cholerae non-O1 NR	1.0
V. damsela FK	1.1
V. fluvialis FK	1.0
V. fluvialis NR	1.0
V. furnissii NR	1.0
V. harveyi FK	1.2
V. vulnificus FK	1.0
Aeromonas hydrophila A 0111003	1.0
Bacillus cereus JCM 2152	1.0
B. subtilis JCM 1465	1.0
Campylobacter jejuni JCM 2013	1.1
Enterobacter aerogenes IFO 13534	1.0
Escherichia coli IFO 3301	1.0
Lactobacillus lactis JCM 1248	1.0
Pseudomonas fragi IFO 3458	1.0
Salmonella enteritidis IFO 3313	1.1
S. typhimurium IFO 12529	1.0
Staphylococcus aureus IFO 3060	1.1

[a] Bacteria (about 10^5 cells) were cultured in AG medium (7 ml) at 37°C for 6 h, and the trypsinlike activity was measured by the fluorogenic assay.

excitation wavelength was 360 nm, and fluorescence intensity was measured at 450 nm. As a control, the culture medium without bacterial cells was similarly assayed. Trypsinlike activity was expressed as the fluorescence intensity of the test sample relative to that of the control sample, which was set at 50. The procedure of the fluorogenic assay is summarized in Fig. 1.

Preparation of seafood samples (5). Five grams of each commercial seafood listed in Tables 2 and 3 was homogenized with 45 ml of peptone water in a sterile Waring blender (14,700 rpm for 2 min) and used as a sample suspension.

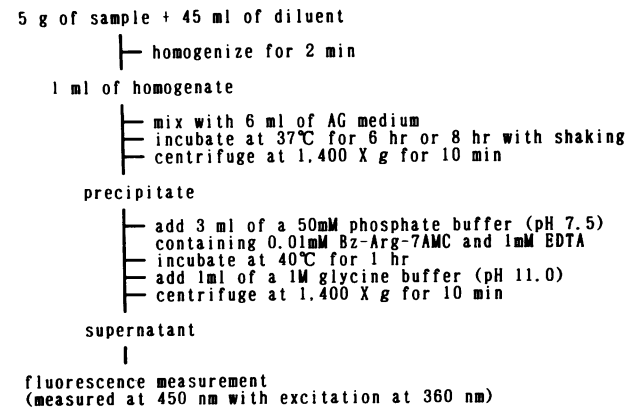

FIG. 1. Procedure of fluorogenic assay for detection of *V. parahaemolyticus* in food.

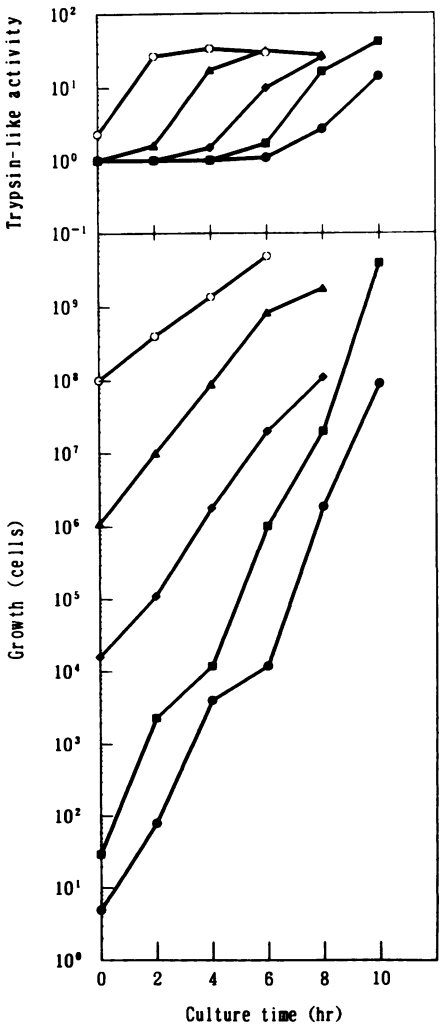

FIG. 2. Time courses of growth and trypsinlike activity of *V. parahaemolyticus* in AG medium. *V. parahaemolyticus* was inoculated into AG medium at various cell numbers and cultured at 37°C. Cell number and trypsinlike activity were measured periodically. Initial cell numbers were 5 (●), 30 (■), 1.5×10^4 (◆), 1.1×10^6 (▲), and 1.0×10^8 (○).

Conventional *Vibrio* detection (5). The sample suspension was serially diluted with peptone water, and then 0.1 ml of the diluted sample was spread evenly over bromothymol blue (BTB) Teepol agar (Nissui Pharmaceutical Co., Ltd.). After incubation at 35°C for 18 h, the typical blue-green colonies of *V. parahaemolyticus* as well as other bacteria were counted.

Five-tube most-probable-number (MPN) analyses of serially diluted samples were done with salt polymyxin broth (Nissui Pharmaceutical Co., Ltd.). Material from tubes showing growth after 24 h at 35°C was streaked on thiosulfate-citrate-bile salts-sucrose agar (Eiken Chemical Co., Ltd.) and cultured for 18 h at 35°C. Typical bluish green colonies were picked up and confirmed as *V. parahaemolyticus* by the following biochemical criteria: triple sugar iron reaction, ability to decarboxylate lysine, and ability to produce indole.

The lower limits of detection of the plating assay and the MPN method are 100 and 1.8 cells per gram, respectively.

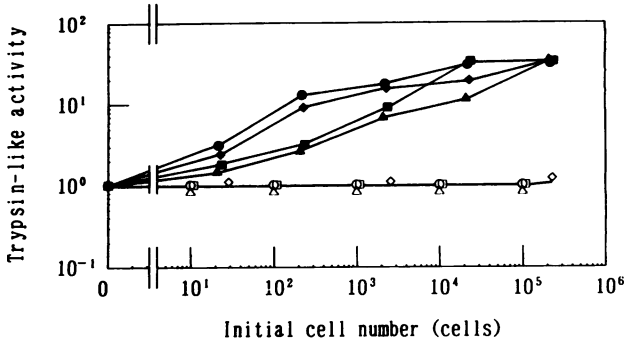

FIG. 3. Trypsinlike activity of various *V. parahaemolyticus* and *V. alginolyticus* strains grown in AG medium. *V. parahaemolyticus* and *V. alginolyticus* were inoculated in AG medium (7 ml) at various cell numbers and cultured at 37°C for 6 h. After centrifugation, the trypsinlike activity of the precipitate was measured by the fluorogenic assay. Symbols: ■, *V. parahaemolyticus* WP-1; ●, *V. parahaemolyticus* 27-2; ▲, *V. parahaemolyticus* 39-3; ◆, *V. parahaemolyticus* 33-8; ○, *V. alginolyticus* 10-1; △, *V. alginolyticus* 13-2; □, *V. alginolyticus* 14-2; ◇, *V. alginolyticus* 14-1.

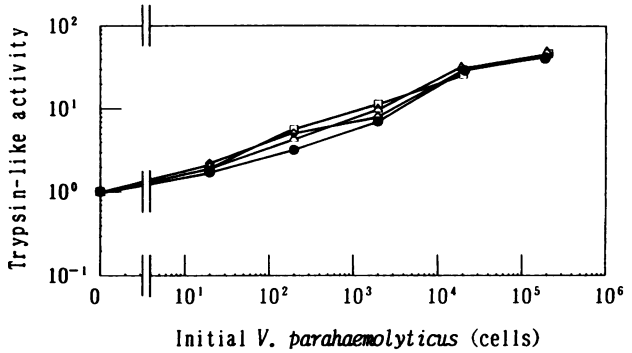

FIG. 4. Trypsinlike activity of *V. parahaemolyticus* grown in the presence of *V. alginolyticus*. *V. parahaemolyticus* WP-1 was cultured in AG medium (7 ml) at 37°C for 6 h in the presence of *V. alginolyticus* 10-1. After centrifugation, the trypsinlike activity of the precipitate was measured by the fluorogenic assay. Symbols: ●, absence of *V. alginolyticus*; △, presence of 3×10^3 cells of *V. alginolyticus*; □, presence of 3×10^4 cells of *V. alginolyticus*; ◇, presence of 3×10^5 cells of *V. alginolyticus*.

RESULTS

Usefulness of AG medium for selective enrichment of *V. parahaemolyticus*. We used tryptone (Difco Laboratories, Detroit, Mich.)-sodium chloride-polymyxin-hexametaphosphate medium (6a) for enrichment of *V. parahaemolyticus* for the fluorogenic assay of the intracellular trypsinlike activity. Since *V. alginolyticus* and *V. harveyi*, as well as *V. parahaemolyticus*, grew vigorously and had high trypsinlike activity in this medium, it was impossible to detect only *V. parahaemolyticus* specifically. To overcome this low specificity, AG medium was developed as a new selective enrichment medium for *V. parahaemolyticus*. The specificity of the medium for selective enrichment of *V. parahaemolyticus* was investigated after a 6-h cultivation of various bacteria at 37°C. Trypsinlike activities of various bacteria in AG medium are shown in Table 1. Of 20 species tested, only *V. parahaemolyticus*, regardless of its Kanagawa response, had high trypsinlike activity in AG medium. The activity of other species, including *V. alginolyticus* and *V. harveyi*, was negligible even when the initial cell number was 10^5.

Time courses of growth and trypsinlike activity of *V. parahaemolyticus*. To determine the minimum incubation period for the detection of trypsinlike activity, 5 to 10^8 cells of *V. parahaemolyticus* WP-1 were cultured in AG medium (7 ml). The time courses of growth and trypsinlike activity are shown in Fig. 2. With the inoculation of five cells of *V. parahaemolyticus*, the cell number increased to about 10^6 and enzyme activity was detected after cultivation for 8 h. With the inoculation of 30 cells, activity was detected after a 6-h cultivation.

Trypsinlike activities of various strains of *V. parahaemolyticus* and *V. alginolyticus* cultured in AG medium. Four strains each of *V. parahaemolyticus* and *V. alginolyticus* were separately inoculated into AG medium, and trypsinlike activity was measured after a 6-h cultivation. The activity was detected in all the strains of *V. parahaemolyticus* when the initial cell number was over 20 (Fig. 3). However, it was not detectable in *V. alginolyticus* even when the initial cell number was 10^5. The trypsinlike activity of *V. parahaemolyticus* is shown in Fig. 4; these organisms were grown in mixed culture with *V. alginolyticus*. The detection of trypsinlike activity of *V. parahaemolyticus* was not interfered with by the presence of 3×10^5 cells of *V. alginolyticus*.

Therefore, AG medium was considered a suitable enrichment medium for *V. parahaemolyticus*, and under the culture conditions used, reliable measurement of trypsinlike activity of *V. parahaemolyticus* was obtained even if a large population of *V. alginolyticus* contaminated the same sample.

Comparison between the improved fluorogenic assay and conventional assays. The detection of *V. parahaemolyticus* in commercial seafoods inoculated with *V. parahaemolyticus* was performed by the improved fluorogenic assay and conventional assays. Fifty contaminated samples of 14 seafoods were examined for *V. parahaemolyticus* by the fluorogenic assay after enrichment cultivation for 6 or 8 h in AG medium. The results were compared with those obtained by the conventional BTB Teepol agar assay (Table 2) and MPN method (Table 3). Trypsinlike activity of *V. parahaemolyticus* in seafood increased in proportion to the initial *V. parahaemolyticus* cell number in the seafood, no matter how many other bacteria, including *V. alginolyticus*, were present (Table 2). Figure 5 shows the relationship between the cell number of *V. parahaemolyticus* in commercial seafood listed in Tables 2 and 3 and trypsinlike activity measured by the fluorogenic assay. Cell number and trypsinlike activity were strongly correlated. After 6- and 8-h enrichments, the correlation coefficients were 0.95 and 0.93, respectively. The fluorogenic assay can detect 10 cells per gram of sample after a 10-h total detection time.

DISCUSSION

By using the arabinose-degrading ability of *V. parahaemolyticus*, Horie et al. (3, 4) developed a modified arabinose-ammonium-sulfate-cholate medium as a selective isolation medium for *V. parahaemolyticus*. *V. parahaemolyticus* was detectable in this medium after incubation at 42°C for 18 h, even if cells of *V. alginolyticus* were 100 times as abundant as those of *V. parahaemolyticus*. Murakami et al. (7) also reported the colony count of *V. parahaemolyticus* with the MPN method in arabinose-colistin-peptone water. However, because some strains of *V. parahaemolyticus* cannot metabolize arabinose (1), non-arabinose-degrading strains of *V.*

TABLE 2. Detection of *V. parahaemolyticus* in seafood by fluorogenic assay and BTB Teepol agar assay

Seafood	Trypsinlike activity (fluorogenic assay)[a]	Cell counts with BTB Teepol agar assay (cells/g)	
		V. parahaemolyticus	Other bacteria
Control	1.0	0	0
Blanquillo	0.9	<100	<100
	1.6	100	<100
	1.9	100	400
	12.5	800	<100
	29.9	9,000	200
Boiled octopus	0.9	<100	<100
	1.0	<100	<100
	8.0	200	<100
Bonito	2.9	100	300
	3.0	100	300
	13.8	1,100	100
	32.3	11,400	<100
Butterfly bream	25.1	9,200	78,400
	34.9	16,400	93,200
	27.8	18,400	62,400
	28.2	18,800	93,200
	41.6	40,000	96,000
Flatfish	5.8	200	<100
	39.0	10,900	<100
Hairtail	1.3	<100	<100
	0.9	<100	<100
Horse mackerel	0.8	<100	300
	1.2	<100	2,200
	2.9	100	3,100
	6.6	1,200	2,000
	32.5	5,900	5,100
Razor clam	7.2	300	13,500
	5.9	500	49,300
	9.7	800	2,700
Sardine	13.4	2,300	1,100
Short-neck clam	15.0	2,000	226,000
Turbot	2.2	100	20,000
	7.1	700	18,600

[a] Trypsinlike activity was measured after a 6-h enrichment cultivation.

TABLE 3. Detection of *V. parahaemolyticus* in seafood by fluorogenic assay and MPN method

Seafood	Trypsinlike activity (fluorogenic assay)[a]	Cell counts with MPN method (*V. parahaemolyticus* cells/g)
Control	1.0	0
Horse mackerel	1.3	7
	3.9	46
Oyster	1.9	7
	5.6	50
	27.2	900
Short-neck clam	1.3	2
	2.8	8
	6.4	110
	18.2	600
Shrimp	4.7	31
	23.3	140
	43.3	370
Yellowtail	7.0	33
	27.5	100
	47.5	500
	48.8	1,130
	48.8	2,000

[a] Trypsinlike activity was measured after an 8-h enrichment cultivation.

parahaemolyticus may remain undetected in these media. Therefore, in the present study, glucuronate was also included in the enrichment medium.

V. fluvialis, *V. harveyi*, and *V. anguillarum* are expected to grow in AG medium and interfere with *V. parahaemolyticus* detection because they can metabolize arabinose or glucuronate (1, 9, 12). The growth of some species of the genera *Pseudomonas* and *Aeromonas*, which are dominant in marine microflora and able to utilize arabinose (1, 10, 11, 12) and which interfere with the growth of gram-negative bacteria, is suppressed at 0.04 to 0.75 μg of polymyxin B per ml (13). Thus, growth of *Pseudomonas* and *Aeromonas* spp. was prevented by including 2 μg of polymyxin B per ml in the medium without inhibiting the growth of *V. parahaemolyticus* (Table 1). The growth of *V. parahaemolyticus* is inhibited by polymyxin B only at concentrations over 2.5 μg/ml (6).

According to Dupray and Cormier (2), 8 h of incubation at 37°C in alkaline peptone water (2% peptone, 3% NaCl, pH 8.6) is best for the isolation of *V. parahaemolyticus* because longer incubation periods may promote the growth of other marine bacteria. Because of the fast growth of *V. parahaemolyticus* on the enrichment medium, the rapid detection of this species was possible even with a small initial number of cells (10 to 10^2) (Fig. 2 through 4). With a longer incubation

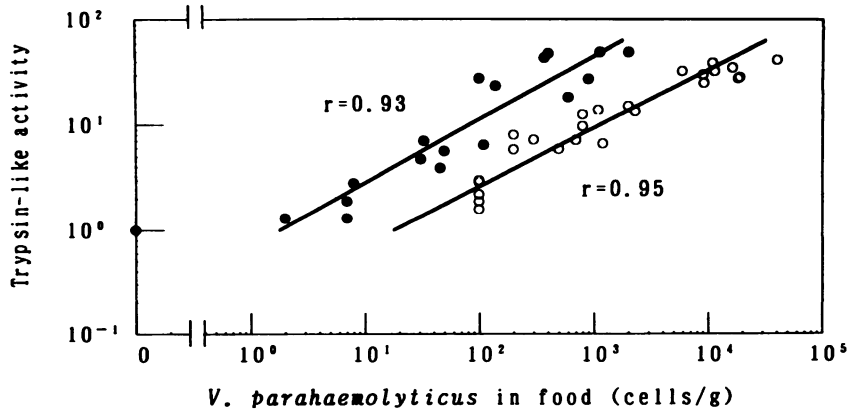

FIG. 5. Relationship between cell number of *V. parahaemolyticus* in seafood and trypsinlike activity measured by the fluorogenic assay. Symbols: ○, after a 6-h cultivation; ●, after an 8-h cultivation.

period, bacteria other than *V. parahaemolyticus* may also grow in AG medium and interfere with the measurement of trypsinlike activity.

Therefore, the assay described in this paper can be used as a highly sensitive and rapid detection method for *V. parahaemolyticus* in the quality control of seafood. Samples with a trypsinlike activity >2 should be considered contaminated with *V. parahaemolyticus*.

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