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Original Article

An Enrichment Medium for Increasing a Very Small Number of *Vibrio parahaemolyticus* Cells to the Detection Limit of the Loop-Mediated Isothermal Amplification (LAMP) Assay

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SUMMARY: We developed an enrichment medium for use with the loop-mediated isothermal amplification (LAMP) assay (enrichment media + LAMP assay) to quickly increase a very small number of *Vibrio parahaemolyticus* cells to the detection limit of the assay. Thirty-nine different enrichment media were prepared based on evaluating 12 potential ingredients. From our assessment of the 39 media, enrichment medium #36, which contained 2% sodium chloride, 1% proteose peptone no. 2, 0.1% trehalose, 0.5% α-ketoglutaric acid, 0.25% pyruvic acid, and 0.5% yeast extract (pH 8.6), was found to be most effective at enhancing the proliferation of *V. parahaemolyticus* during incubation for 3 h at 40°C. We compared the detection limits of the LAMP assay, the enrichment medium #36 + LAMP assay, and the cultivation method using bacterial cell and spiked shrimp sample tests. The detection limits of the LAMP assay, the medium #36 + LAMP assay, and the cultivation method were 10^3, 10^0–10^{-1}, and 10^{-1} CFU ml^{-1}, respectively. Enrichment medium #36 promoted a 10^3- to 10^4-fold increase in the bacterial population, and the detection limit of the enrichment media + LAMP assay was the same as that of the cultivation method.

INTRODUCTION

Vibrio parahaemolyticus exists naturally in estuarine waters and is often isolated from coastal waters, as well as from sediment, suspended particles, plankton, and a variety of fish and shellfish. This pathogen causes gastroenteritis, which is almost exclusively associated with consuming raw seafood and seafood that has been inadequately cooked or cooked but then recontaminated (1,2).

In Japan, *V. parahaemolyticus* is an important causative agent of food-borne gastroenteritis outbreaks due to the custom of eating raw seafood. Although the number of patients involved in such incidents has gradually decreased from 12,318 in 1998 to 1,278 in 2007, large-scale outbreaks still occur. For example, a diffuse outbreak caused by squid that had been salted using a low salt concentration was reported in 2007. This case involved 620 patients spread across the Tokyo metropolis, 5 prefectures, and 6 cities (3).

V. parahaemolyticus produces a thermolabile hemolysin (TLH), and pathogenic strains produce a thermostable direct hemolysin (TDH), and/or a TDH-related hemolysin (TRH). The TDH molecule has been extensively studied and is most closely linked to the onset of disease (4). Interestingly, *tdh* gene-positive strains are mostly isolated from diarrhea patients, but such strains are rarely detected in seawater or seafood (1).

To detect *V. parahaemolyticus* in seafood, a cultivation method involving the use of alkaline peptone water (AP) and thiosulfate citrate bile sucrose (TCBS) agar has been developed. This method has considerable sensitivity, but it requires 3 or more days to identify the species. Recently, molecular techniques such as the loop-mediated isothermal amplification (LAMP) assay, PCR, and real time-PCR have been developed to target toxin genes like *tdh* or *trh*. In particular, the LAMP assay is rapid, as well as being easier to perform (5–7).

In a previous study, we developed a *tdh*-targeting LAMP assay for detecting *V. parahaemolyticus* in seafood with considerable specificity and sensitivity (8). Although the LAMP assay is a rapid and easy tool, it is less able to detect *V. parahaemolyticus* than routine cultivation methods. In order to increase the ability of the LAMP assay to detect *V. parahaemolyticus* in seafood, we are planning to perform a short period of enrichment before the LAMP assay (enrichment medium + LAMP assay). In this study, we attempted to develop an enrichment medium that is able to increase the number of *V. parahaemolyticus* cells to a level above the detection

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limit of the LAMP assay during 3 h of incubation.

MATERIALS AND METHODS

Bacterial strains: A clinically isolated *V. parahaemolyticus* 1052A strain was used in this study. The clinical strains, 1011 and 1038, were also used. These strains were identified by means of biochemical and serological examinations. All of the strains were found to be *tdh* gene-positive using a conventional PCR assay (8) and were serotyped as O3:K6.

Preparation of the enrichment media: A total of 12 ingredients from 3 goups were examined: (i) peptones (peptone, proteose peptone no. 2, soytone, and tryptose); (ii) sugars (glucose, mannitol, and trehalose); and (iii) others (α-ketoglutaric acid, pyruvic acid, lactalbumin, beef extract, and yeast extract). The α-ketoglutaric acid and pyruvic acid were obtained from MP Biomedicals, LLC. (Solon, Ohio, USA). The lactalbumin and the 3 sugars were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other ingredients were purchased from Becton, Dickinson and Company (Sparks, Md., USA). The sugars and the ingredients classified as ''others'' were added to AP at final concentrations of 0.2 and 1%, respectively. Peptones were used to replace the polypeptone in the AP at a final concentration of 1%. The pH of these media was adjusted to 8.6. Then, 100-μl aliquots of *V. parahaemolyticus* overnight culture dilution were added to each 1.9-ml sample of AP (the resultant mixture contained 10^2 to 10^4 CFU ml^{-1}), and the mixture was incubated for 3 h at 37°C. The 3-h incubation was used to obtain results within 1 day. The number of CFU ml^{-1} was counted after plating 100 μl of the culture dilution on a tryptosoy agar plate (Eiken Chemical Co., Ltd., Tochigi, Japan) containing 1.5% sodium chloride. Relative

Table 1. Compositions and ingredient concentrations (W/W %) of the 39 enrichment media

Medium	NaCl	Pep	PP	Glc	Man	Tre	α-KA	LA	PA	YE
1	2	2								
2	2	1		0.2				1		0.5
3	2	1			0.2					
4	2	1				0.2				
5	2	1				0.1				
6	2	1					3			
7	2	1					2			
8	2	1					1.5			
9	2	1					1			
10	2	1					0.5			
11	2	1					0.1			
12	2	1						1		1
13	2	1							3	
14	2	1							2	
15	2	1							1.5	
16	2	1							1	
17	2	1							0.5	
18	2	1							0.1	
19	2	1							0.25	
20	2	1								1
21	2	1								0.5
22	2		2		0.2					1
23	2		2		0.2					
24	2		2							1
25	2		2							
26	2		1.5	0.3				1		1
27	2		1.5		0.2					0.5
28	2		1.5			0.2	1		0.5	0.5
29	2		1.5			0.2	1			0.5
30	2		1.5			0.2		1		1
31	2		1.5			0.2			0.5	0.5
32	2		1.5			0.2				0.5
33	2		1.5					1		1
34	2		1.5							0.5
35	2		1.5							
36	2		1			0.1	0.5		0.25	0.5
37	2		1					1		1
38	2		1							0.5
39	1.5		1.5		0.1					

Pep, peptone; PP, proteose peptone no. 2; Glc, glucose; Man, mannose; Tre, trehalose; α-KA, α-ketoglutaric acid; LA, lactalbumin; PA, pyruvic acid; YE, yeast extract.

growth rate (e.g., CFU ml^{-1} produced by the AP + test ingredient/CFU ml^{-1} produced by AP alone) was used to evaluate the effectiveness of each ingredient. The data are presented as mean ± standard deviation values of 3 experiments performed on different days.

Evaluations of prepared enrichment media and cultivation temperature: Thirty-nine enrichment media were prepared based on various combinations of the 9 selected ingredients. Table 1 shows the ingredients used and their concentrations (W/W %) in the 39 enrichment media. The pH of the media was adjusted to 8.6. The relative growth rate (CFU ml^{-1} produced by the enrichment medium/CFU ml^{-1} produced by AP) was measured after incubating *V. parahaemolyticus* in each medium for 3 h at 37°C. The data were obtained from a single experiment. The culture temperature was determined after testing 4 different temperatures (30, 37, 40, and 42°C) using the 2 most useful enrichment media. The data presented are mean ± standard deviation values for at 3 experiments performed on different days.

Comparison of the sensitivities of the LAMP assay, the enrichment media + LAMP assay, and the cultivation method: All media contained 10^{-2} to 10^{3} CFU ml^{-1} of *V. parahaemolyticus* strain 1052A. The frozen *V. parahaemolyticus* 1052A stock was cultured overnight on a trypto-soy agar plate containing 1.5% sodium chloride. One colony from the plate was suspended in 3 ml of PBS buffer, which resulted in an approximate concentration of 10^{8} CFU ml^{-1}. The suspension was then serially diluted 10-fold, and 100 μl of the dilution were added to 1.9 ml of AP, an enrichment medium, or a spiked shrimp sample, resulting in *V. parahaemolyticus* concentrations of 10^{3} to 10^{-2} CFU ml^{-1}. The spiked shrimp sample was prepared as follows by using different lots of shrimp: commercially available shrimp was cut into pieces, and a 10-g sample was inoculated into 90 ml of AP or enrichment medium and then was well homogenized. Then, a 1.9-ml sample of the mixture was used for the subsequent experiment.

LAMP assay: Fifty microliters of bacterial culture were mixed with an equal volume of sodium hydroxide solution (25 m mol l^{-1}) in a 1.5-ml microcentrifuge tube, and the mixture was heated at 95°C for 15 min. After adjusting the pH with 8 μl of Tris-HCl buffer (1 mol l^{-1}, pH 7.5), cell debris was pelleted by centrifugation at 20,000 g and 4°C for 5 min, and 2 μl of the resulting supernatant were used as template DNA. The LAMP assay was carried out using a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan), as described previously (8). Briefly, 25 μl of the reaction mixture contained 40 pmol each of the BIP (5′-atggctgacatcctacatgacttgcttatagccagacacc-3′) and FIP primers (5′-ctgacgttgtgaatactgattgaccggtctctgacttttgga ca-3′), 20 pmol each of the LB (5′-aagactatacaatggcagc-3′) and LF primers (5′-tggcatgtttctaca-3′), 5 pmol each of the B3 (5′-acgaacacagcagaatgac-3′) and F3 primers (5′-caatgcaccggtcaatgt-3′), 1 μl of *Bst* DNA polymerase, and 2 μl of template DNA. The reaction mixture was incubated at 60°C for 60 min. The reaction was terminated by heating at 80°C for 2 min, and the samples were cooled to 4°C. The turbidity at 650 nm was monitored consecutively using a RT-160C turbidimeter (Eiken Chemical).

Enrichment medium + LAMP assay: Enrichment media containing 10^{3} to 10^{-2} CFU ml^{-1} of *V. parahaemolyticus* were incubated for 3 h at 40°C. The LAMP assay was then performed as described above.

Cultivation method: AP containing 10^{3} to 10^{-2} CFU ml^{-1} of *V. parahaemolyticus* was incubated at 37°C overnight. Then, a 100-μl aliquot of the culture was spread onto a TCBS agar plate and incubated at 37°C overnight. Characteristic green colonies on the TCBS agar plate were subjected to biochemical and serological analyses to verify that they were *V. parahaemolyticus* colonies.

Statistical analyses: Statistical analyses of the data including one-way analysis of variance (ANOVA) were carried out using GraphPad Prism version 4.03 (GraphPad software). Differences were considered statistically significant when the relevant P value was <0.05.

RESULTS

Evaluations of the enrichment media and culture temperature: (i) Effects of the ingredients: Relative growth rate was measured for 12 ingredients (Fig. 1). Of the 4 peptones, proteose-peptone no. 2 enhanced the proliferation of *V. parahaemolyticus* the most, resulting in a relative growth rate value of 1.3 ± 0.4 (Fig. 1, PP). All of the 3 sugars tested scored relatively high relative growth rates. As for the "others," yeast extract showed the highest score (3.17 ± 2.7), followed by α-ketoglutaric acid and pyruvic acid, which displayed relative growth rates of 2.1 ± 1.28 and 1.91 ± 1.46, respectively. However, none of the ingredients produced significantly high relative growth rate values compared with the 1.0 of the AP.

(ii) Enrichment media and culture temperature: As no effective growth enhancers were found among the 12 ingredients tested, we prepared 39 AP-based enrichment media containing 1 to 5 ingredients in addition to sodium chloride (Table 1). Sodium chloride is an essential reagent for *V. parahaemolyticus* growth and was included in all of the media. The concentration at which it was added (2%) was sufficient for *V. parahaemolyticus* growth (data not shown). A preliminary examination of

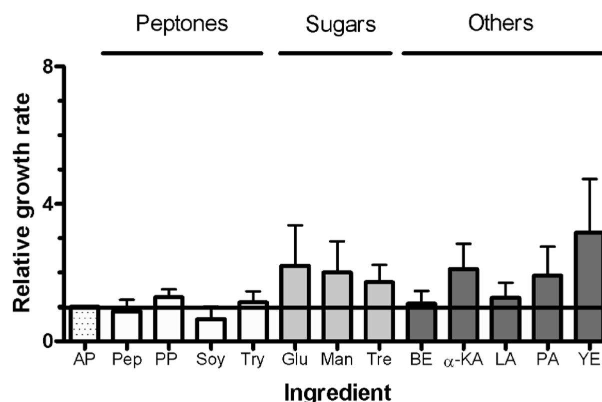

Fig. 1. Relative growth rate of the 12 ingredients. AP, alkaline peptone water; Pep, peptone; PP, proteose peptone no. 2; Soy, soytone; Try, tryptose; Glc, glucose; Man, mannose; Tre, trehalose; BE, beef extract; α-KA, α-ketoglutaric acid; LA, lactalbumin; PA, pyruvic acid; YE, yeast extract. The horizontal line in the figure indicates the relative growth rate of AP (1.0).

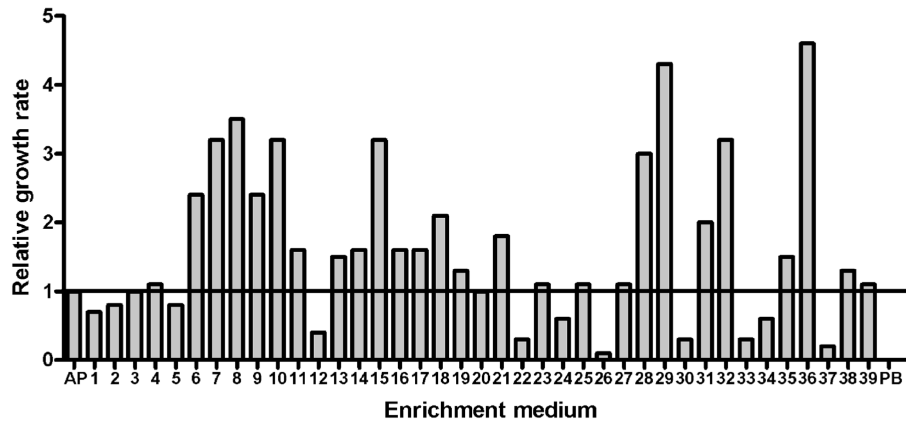

Fig. 2. Relative growth rate of 39 enrichment media in addition to those of AP (alkaline peptone water) and PB (salt polymyxin broth; NISSUI Pharmaceutical, Co., Ltd., Tokyo, Japan). The horizontal line in the figure indicates the relative growth rate of AP (1.0).

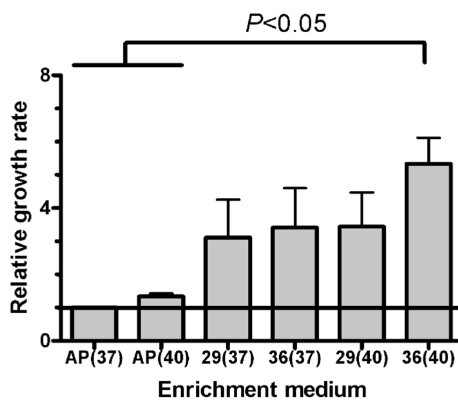

Fig. 3. Effect of culture temperature on the relative growth rate of enrichment media #29 and #36. The horizontal line in the figure indicates the relative growth rate of AP (1.0). The figures in parentheses indicate the culture temperature.

all of the 39 media revealed that 2 media (#29 and #36) scored relatively high relative growth rate values of 4.3 and 4.6, respectively (Fig. 2).

Culture temperature was assessed using these 2 media (#29 and #36). At 30 and 42°C, no growth was detected in either medium (data not shown). When we compared the relative growth rate of the media between 37 and 40°C, medium #36 exhibited an increased relative growth rate at 40°C (Fig. 3), as did medium #29. Medium #36 displayed higher scores than medium #29 at both temperatures. Finally, at 40°C medium #36 displayed a significantly increased relative growth rate, compared with AP at 37 and 40°C ($P < 0.05$). In addition, the 2 clinical O3:K6 strains containing the *tdh* gene proliferated as readily as the 1052A test strain on medium #36.

Detection limits of the LAMP assay, the enrichment medium #36 + LAMP assay, and the cultivation method: Table 2 shows the detection limits of the LAMP assay, the enrichment medium #36 + LAMP assay, and the cultivation method, which were obtained from bacterial cell tests and spiked shrimp sample tests. For the bacterial cell tests, the LAMP assay produced positive results in 3 tests at 10^3 CFU ml^{-1}, while a conflicting result was obtained at 10^2 CFU ml^{-1} (i.e., the detection limit of the LAMP assay was 10^3 CFU ml^{-1}). The medium #36 + LAMP assay was able to detect the bacteria in 3 samples at concentrations as low as 10^{-1} CFU ml^{-1}, and in 2 out of 3 tests at a concentration of 10^{-2} CFU ml^{-1}. To confirm the presence of viable cells, dilutions of the medium #36 + LAMP-positive samples with concentrations ranging from 10^{-1} to 10^{-2} CFU ml^{-1} were plated on trypto-soy agar containing 1.5% sodium chloride. The number of bacterial cells ranged from 1.1×10^3 to 1.2×10^4 CFU ml^{-1} in 5 samples. As for the spiked shrimp sample tests, the detection limit of the LAMP assay was 10^3 CFU ml^{-1}, which was the same as the result for the bacterial cell test, but positive results were still obtained at 10^1 CFU ml^{-1}. The detection limit of the medium #36 + LAMP assay was 10^0 CFU ml^{-1}, while a positive result was even obtained at 10^{-2} CFU ml^{-1}. Green colonies of 4.0×10^1 to 3.7×10^2 CFU ml^{-1} were produced by the medium #36 + LAMP-positive samples at 10^{-1} and 10^{-2} CFU ml^{-1} after plating on TCBS agar. In this study, the use of enrichment medium #36 promoted a 10^3–10^4-fold increase in the cell count, enabling the LAMP assay to detect 10^{-1}–10^0 CFU ml^{-1} of the bacteria.

Finally, we compared the detection limits of the enrichment medium #36 + LAMP assay and the cultivation method. The detection limit of the medium #36 + LAMP assay was 10^{-1} CFU ml^{-1}, which was the same as that of the cultivation method for the bacterial cell test. However, the detection limit of the medium #36 + LAMP assay was 10-fold lower than that of the cultivation method for the spiked shrimp sample.

DISCUSSION

Of all the media we tested, enrichment medium #36 was the most useful medium for increasing the proliferation of *V. parahaemolyticus* during a 3-h incubation at 40°C, and thus, is suitable for combination with the LAMP assay.

One of advantages of pre-incubation using enrichment medium #36 is that the detection limit of the medium #36 + LAMP assay is similar to that of the cultivation method. In other words, this medium achieved a 10^3- or 10^4-fold increase in the bacterial cell to the detection limit of the LAMP assay. Use of enrichment medium #36 with the LAMP assay dramatically decreased

Table 2. Comparison of the sensitivities of three detection methods

		CFU ml^{-1}					
		10^3	10^2	10^1	10^0	10^{-1}	10^{-2}
Bacterial cell test	LAMP	+	± (2/3)[1)]	−	−	ND	ND
	#36+LAMP	ND	+	+	+	+	± (2/3)
	Cultivation	+	+	+	+	+	−
Spiked shrimp-sample test	LAMP	+	± (2/3)	± (1/3)	−	ND	ND
	#36+LAMP	ND	+	+	+	± (2/3)	± (1/3)
	Cultivation	+	+	+	+	+	−

1): The results of the three examinations were not identical (No. of positive results/No. of tests).
ND, not done.

the detection time from at least 3 days to only 5 h. These advantages make this method suitable for routine surveillance for *V. parahaemolyticus* in seafood. However, it still remains to be determined whether the *tdh* gene is an appropriate target for detecting *V. parahaemolyticus* because it is predominately found in strains obtained from diarrhea patients, rather than in those obtained from seafood or the environment. Thus, there is a concern that the results of the LAMP assay are not equivalent to those produced by the cultivation method. In spite of this concern, the detection of *tdh* gene-positive *V. parahaemolyticus* is significant for 2 reasons: (i) Identification of *tdh* gene-positive *V. parahaemolyticus* in commercially available seafood may be important with regard to minimizing the risk of a food-borne outbreak. (ii) In the food-borne outbreaks caused by *V. parahaemolyticus*, a *tdh* gene-positive strain has easily been isolated from diarrhea patients; however, it is rarely isolated from a suspected food. If the medium #36 + LAMP assay is able to detect low levels of *tdh* gene-positive *V. parahaemolyticus* in the food, it would support epidemiological information (e.g., presumption of the suspected food by means of epidemiological analysis). In addition, we should develop different primer sets for detecting other genes that are specific to *V. parahaemolyticus*. Possible candidates include the *gyrB* and *toxR* genes. The *gyrB* gene encodes the B subunit of DNA gyrase. A PCR procedure targeting the *gyrB* gene has been developed for the specific detection of *V. parahaemolyticus* in shrimp (9). The *toxR* gene is well conserved among *Vibrio* spp. and regulates many virulence factor genes. A *toxR* gene-targeting PCR protocol has also been established for the specific detection of *V. parahaemolyticus* (10).

The application of medium #36 to the LAMP assay enabled the detection *V. parahaemolyticus* at a concentration of 10^{-2} CFU ml^{-1} in the tests. This result was not a false positive because: (i) the presence of viable bacteria was verified by means of plating after 3-h incubation. The *V. parahaemolyticus* proliferated to just below the detection limit of the LAMP assay, which rules out the possibly of the detected DNA having been released from dead cells. (ii) Gel electrophoresis of the reaction mixture after the LAMP reaction confirmed the expected DNA fragment, not a non-specific DNA fragment. It is possible that medium #36 is superior in terms of recovering injured bacteria because it contains α-ketoglutaric acid and pyruvic acid, which have not been included in any previously reported enrichment broth for *V. parahaemolyticus* (11). These reagents are used as supplements in the enrichment medium for *Campylobacter* spp. and are thought to prevent the generation of peroxides like H_2O_2, which are harmful to bacteria. Of the top 10 enrichment media in the 39 media evaluated, 9 contained α-ketoglutaric acid, pyruvic acid, or both; however, it currently remains unclear whether these reagents aided the recovery of *V. parahaemolyticus* that were injured or subjected to temperature stress at 40°C.

In summary, enrichment medium #36, which contained 2% sodium chloride, 1% proteose peptone no. 2, 0.1% trehalose, 0.5% α-ketoglutaric acid, 0.25% pyruvic acid, and 0.5% yeast extract (pH 8.6), was the medium that was most able to increase *V. parahaemolyticus* proliferation during 3-h pre-incubation at 40°C.

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Conflict of interest None to declare.

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