表8. 魚貝類由来株および食中毒患者由来株のPFGE解析の結果

Sero type	PFGE(Not1)	PFGE(Sfi1)	tdh	trh	GS	ORF8	魚貝類由来株	食中毒患者由来株
O3:K6	3	a	+	-	+	+	岩がき(2001年), 国産アサリ(2008年)	2007年
		e	+	-	+	+	国産アオヤギ(2001年)	2006年
		h	+	-	+	+		1998年
	2	a	+	-	+	+	国産岩がき(2001年), 韓国産アカガイ(2009年)	1998, 2005、2006年
		c	+	-	+	+		2001年
		f	+	-	+	+		2007年
		b	+	-	+	-		2007年
	5, 5'	a'	+	-	+	+	国産アオヤギ(2001年)	
		e	+	-	+	+	国産岩がき(2001年)	
		g	+	-	+	-		2001年
	7, 7'	a'	+	-	+	+	中国産アカガイ(2009年)	
		d	+	-	+	+		2008年
	10	a'	+	-	+	+	国産アサリ(2008年)	2007年
		f	+	-	+	+		2005年
	1, 1'	a	+	-	+	+		2006, 2007, 2008年
	4		+	-	+	+	国産アサリ(2001年)	
	6		+	-	+	+		1999年
	8		+	-	+	+		1998-2001年
	9		+	-	+	+	国産アサリ(2008年)	
	11		+	-	+	+	中国産アカガイ(2009年)	
	12	a	+	-	+	+		2007年
	13		+	-	+	+		2006年
	14		+	-	+	+		2007年
	15		-	-	-	-	国産アサリ(2008年)	
	16		-	-	-	-	国産アサリ(2007年)	
	17		-	-	-	-	国産アジ(2008年)、国産アサリ(2008年)	
	34		-	-	-	-	国産アジ(2008年)	
	18		-	-	-	-	国産アサリ(2007年)	
	19, 19'		-	-	-	-	国産アオヤギ(2007年)	
	20		-	-	-	-	国産ハマグリ(2007年)	
	21		-	-	-	-	国産アサリ(2007年)	
	22		-	-	-	-	国産アサリ(2007年)	
	23		-	-	-	-	国産アサリ(2007年)	
	24		-	-	-	-	国産アオヤギ(2001年)、国産ハマグリ(2007年)	
	25		-	-	-	-	国産マテガイ(2007年)	
	26		-	+	-	-		2000年
	27		-	-	-	-	国産アサリ(2007年)	
	28		-	-	-	-	国産アサリ(2008年)	
	29		-	-	-	-	国産アサリ(2008年)	
	30		-	-	-	-	国産アサリ(2007年)	
	31		-	-	-	-	国産アジ (2008年)	
	32		-	-	-	-	国産アサリ(2007年)	
	33		-	-	-	-	国産アオヤギ(2007年)	
	27'		-	-	-	-	国産アオヤギ(2007年)	

表8. 魚貝類由来株および食中毒患者由来株のPFGE解析の結果(つづき)

Sero type	PFGE(Not1)	PFGE(Sfi1)	tdh	trh	GS	ORF8	魚貝類由来株	食中毒患者由来株
O1:K25、O1:KUT	1、1'		+	+	—	-		2001, 2007年
	2		+	+	—	-	韓国産アカガイ(2009年)	
	3		+	-	+	-		2001, 2006年
	4		+	-	+	+		2006年
	5		+	-	+	-		2006年
O4:K8	1		+	-	-	-		2000年
	2		+	-	-	-		1997年
	3		+	-	-	-		1998, 1999年
	4		+	-	-	-		2006年
	5		+	-	-	-		2006年
O4:K9	1		+	-	-	-		1999年
	1"		+	+	-	-		2006年
	1	i	+	-	-	-	国産アサリ(2007年)	2007年
O4:K68	1, 1', 1"		+	-	+	+/—		1998, 1999, 2001, 2006年
O10:K52	1, 1'		+	+	-	-	国産アジ(2008年), 国産アサリ(2008年)	
O4:K12	2		+	-	-	-		1998年
	3		+	+	-	-		2006年
O8:K21	4		+	-	-	-		2006年
	5		+	-	-	-	韓国産アカガイ(2009年)	
O4:K11	6		+	-	-	-		1999年
O4:K13	7		+	-	-	-		1999, 2006年
O3:K7	8		+	-	-	-	韓国産アカガイ(2009年)	
O4:K37	9		+	-	-	-	国産アオヤギ(2007年)	
	9		+	-	-	-	国産アオヤギ(2007年)	
OUT:K38	9		+	-	-	-	国産アオヤギ(2007年)	
OUT:KUT	9		+	+	-	-	国産アオヤギ(2007年)	
O9:K44	11		+	-	-	-		2008年
O4:K12	12		-	-	-	-		2006年
O4:K55	13		+	-	-	-		2006年
O4:KUT	14		+	-	-	-	国産アオヤギ(2007年)	
O10:K52,O10:KUT	15		+	+	-	-	国産アジ(2008年),韓国産アサリ(2009年)	
OUT: K37	16		+	-	-	-	国産アオヤギ(2007年)	
OUT:KUT	16		+	+	-	-	国産アオヤギ(2007年)	
O4:KUT	17		+	-	-	-	国産アジ(2008年)	
O5:K17	19		+	+	-	-	国産アサリ(2008年), 韓国産アカガイ(2009年)	

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nemoto, J., Sugawara, C., Akahane, K., Hashimoto, K., Kojima, T., Ikedo, M., Konuma, H., Hara-Kudo, Y.	Rapid and specific detection of the thermostable direct haemolysin gene in *Vibrio parahaemolyticus* by the Loop-mediated isothermal amplification	Journal of Food Protection	72巻	748-754	2009
山崎省吾，右田雄二，中村まき子，浦伸孝，工藤由起子，三澤尚明，岡本嘉六，髙瀬公三	長崎県沿岸における *Vibrio vulnificus* の分布と環境因子	日本獣医師会誌	62巻	649-655	2009
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Rapid and Specific Detection of the Thermostable Direct Hemolysin Gene in *Vibrio parahaemolyticus* by Loop-Mediated Isothermal Amplification

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ABSTRACT

Several investigators have reported that thermostable direct hemolysin (TDH) and TDH-related hemolysin are important virulence factors of *Vibrio parahaemolyticus,* but it has been difficult to detect these factors rapidly in seafood and other environmental samples. A novel nucleic acid amplification method, termed the loop-mediated isothermal amplification (LAMP), which amplifies DNA with high specificity and rapidity under isothermal conditions, was applied. In this study, we designed *tdh* gene-specific LAMP primers for detection of TDH-producing *V. parahaemolyticus.* The specificity of this assay was evaluated with 32 strains of TDH-producing *V. parahaemolyticus,* one strain of TDH-producing *Grimontia hollisae,* 10 strains of TDH-nonproducing *V. parahaemolyticus,* and 94 strains of TDH-nonproducing bacteria, and the sensitivity was high enough to detect one cell per test. Moreover, to investigate the detection of TDH-producing *V. parahaemolyticus* in oysters, the LAMP assay was performed with enrichment culture in alkaline peptone water of oyster samples inoculated with TDH-producing *V. parahaemolyticus* and TDH-nonproducing *V. parahaemolyticus* and *V. alginolyticus* after enrichment in alkaline peptone water. These results suggest that the LAMP assay targeting *tdh* gene has high sensitivity and specificity and is useful to detect TDH-producing *V. parahaemolyticus* in oyster after enrichment.

Vibrio parahaemolyticus is a pathogen that causes foodborne infections associated with the consumption of fish or shellfish worldwide *(4, 9, 10, 22, 23).* Most clinical strains of *V. parahaemolyticus* produce thermostable direct hemolysin (TDH) or TDH-related hemolysin. It is reported that these hemolysin variants are important virulence factors in pathogenic *V. parahaemolyticus (14, 16).* Since TDH and/or TDH-related-hemolysin–producing *V. parahaemolyticus* organisms are present in seafood and the environment at low concentrations and in a small portion of microorganisms' flora, it is difficult to detect them in seafood or environmental samples *(11, 17).* Testing for the Kanagawa phenomenon caused by TDH on Wagatsuma blood agar medium is cumbersome to carry out *(3, 20, 21).* Although an enzyme-linked immunosorbent assay for detecting TDH has been reported *(6),* the compositions of food or clinical samples inhibit the interaction between antigens and antibodies. Using a combination of enrichment and PCR assay, 8 h was required for the detection of pathogenic *V. parahaemolyticus* that had been artificially inoculated in UV-treated oysters *(1, 2).* In 2000, Notomi et al. *(19)* reported a novel nucleic acid amplification method, termed the loop-mediated isothermal amplification of DNA (LAMP). Several researchers have reported that the LAMP assay is a more sensitive and rapid method than PCR assay *(5, 7).* Furthermore, this assay has several advantages over PCR, nested PCR, and real-time PCR assays, as follows. The LAMP reaction is done under isothermal conditions between 60 and 65°C *(8, 13, 19)* using a simple incubator such as a water bath or block heater, which is sufficient for amplification. The reaction is highly specific for the target sequence because the primers recognize six distinct regions on the targeted DNA. The LAMP assay is suitable for detection of target genes both in laboratories and in field situations because LAMP produces a large amount of pyrophosphate ion as a by-product that is utilized to create a turbidity in the reaction mixture visible to the naked eye *(12).* Finally, the reaction procedure is more economical and practical than both PCR and real-time PCR assays.

One of the notable characteristics of *V. parahaemolyticus* is rapid growth under optimum conditions. After a 3-h incubation, the cell numbers rise to approximately 10^6 times the original number. We noted that the number of *V. parahaemolyticus* rises over the detection limit with LAMP assays after a few hours of enrichment in alkaline peptone water. In this study, we designed a set of primers for detecting the *tdh* gene. This is the first report demonstrating the usefulness of the LAMP assay to detect TDH-producing *V. parahaemolyticus.* Detection of TDH-producing *V. parahaemolyticus* in oysters was investigated in combinations of enrichment procedures and LAMP assay in this study.

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MATERIALS AND METHODS

Bacterial strains and culture. The strains used in this study are listed in Table 1. In *V. parahaemolyticus,* 30 strains (1 strain each of serotypes OUT:K5, OUT:K29, OUT:K57, O1:KUT, O1:K1, O1:K25, O1:K56, O4:K12, O4:K13, O5:K68; 2 strains of serotypes O4:K9 and O4:K68; 3 strains of O4:K8; 13 strains of O3:K6) from clinical specimens; 10 strains (1 strain each of serotypes OUT:K22, OUT:K32, O4:K49, O4:K64, O10:K24, OUT:KUT; 4 strains of serotype O3:K6) from food samples; and 2 strains of serotype O3:K6 from the environment were used. One strain of *Grimontia hollisae* (ATCC 33564) was purchased from American Type Culture Collection (Rockville, MD). The strains of *Vibrio* spp. and *G. hollisae* were grown on heart infusion agar medium (Eiken Chemical Co. Ltd., Tokyo, Japan) with 2.0% (wt/vol) NaCl at 37°C for 18 h. The other bacteria were grown on 5% sheep blood agar medium at 37°C for 18 h. All strains were tested for the *tdh* gene by *tdh*-PCR assay *(21).* Forty-two strains of *V. parahaemolyticus* and *G. hollisae* were tested for TDH production by reverse passive latex agglutination (RPLA) assay with a kit (KAP-RPLA; Denka Seiken, Tokyo, Japan). To observe the Kanagawa phenomenon, 40 strains of *V. parahaemolyticus* and *G. hollisae* (Table 1) were tested for hemolytic activity by Wagatsuma blood agar medium *(3).*

Design of primer for LAMP. The LAMP primers were designed based on the published *tdh* sequences in *V. parahaemolyticus* (GenBank accession nos. AP005088, AY044107, AY044108, AY044109, AY044110, AY044111, AY044112, AY044113, AY044114, AY249144, D90100, D90101, D90238, M10069, S67841, S76724, X54340, X54341, X54342, and X54343), *G. hollisae* (GenBank accession no. M57900), *Vibrio mimicus* (GenBank accession no. M64120), and *Vibrio cholerae* non-O1 (GenBank accession no. M55316) (Fig. 1A). The forward inner primer (FIP) consisted of the complementary sequence of F1 (F1c) and F2 (Fig. 1B): 5′-CTTATAGCCAGACACCGCTGCGGTTGACATCCTACATGACTGTG-3′. The backward inner primer (BIP) consisted of B1c, T linker and the B2: 5′-CGGTCATTCTGCTGTGTTCGTTCTTCACCAACAAAGTTAGCTACAG-3′. The forward outer primer (F3) and the backward outer primer (B3) were 5′-GTCTCTGACTTTTGGACAAACCG-3′ and 5′-CTACATTAACAAAATATTCTGGAGTTTCATCC-3′. The forward loop primer (FLP) and the backward loop primer (BLP) were 5′-CCGCTGCCATTGTATAGTCTTT-3′ and 5′-CAGATCAAGTACAACTTCAACATTCCT-3′.

LAMP assay. The LAMP assay was carried out in a total of 25 μl of reaction mixture containing 40 pmol of FIP, BIP, FLP, and BLP, 10 pmol of F3 and B3, 12.5 μl of 2× Universal LAMP reaction mixture (Eiken Chemical Co. Ltd.), 1 μl of *Bst* DNA polymerase, and 5 μl of template DNA. The mixture was mixed gently, and this was incubated at 65°C. The result of the reaction was evaluated at 60 min of incubation. The LAMP reaction synthesized an extremely large amount of DNA, and white precipitation appeared as a by-product *(12).* The increase of turbidity in the reaction mixture correlated with the amount of DNA amplification and was monitored with an LA-320C real-time turbidimeter for LAMP (Eiken Chemical Co. Ltd.).

PCR assay. The PCR assay for the detection of *tdh* gene was performed with primer 1 (5′-GGTACTAAATGGCTGACATC-3′) and primer 2 (5′-CCACTACCACTCTCATATGC-3′) as described by Tada et al. *(21).* The reaction was carried out in a total of 50 μl of reaction mixture containing 9.5 pmol each of the primers, 5 μl of 10× ExTaq-Buffer, 0.2 μM each of the four deoxynucleoside triphosphates, 2.5 U of Ex *Taq* polymerase (Takara Ex Taq, Takara, Ohtsu, Japan), and 5 μl of template solution. The amplification conditions were set at a first cycle of 96°C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by one cycle of 72°C for 7 min. All of the PCR products were confirmed by electrophoresis of 2% agarose gel under 1× Tris-borate-EDTA buffer to recognize the specific amplicon (251 bp).

Specificity of the LAMP and PCR assays. All of the strains listed in Table 1 were suspended in 2.5 % NaCl for the strains of *Vibrio* spp. and *G. hollisae* or 0.85 % NaCl for the other strains at a concentration of McFarland turbidity standard of 1, which is approximately 3.0×10^8 CFU/ml. These suspensions were diluted to approximately 1.2×10^4 CFU/ml in Tris-EDTA (TE) buffer. Then, each dilution was denatured at 95°C for 5 min and centrifuged at 2,000 × *g* for 1 min. Five microliters of each supernatant was added in independent reaction tubes as a LAMP template and a PCR template. The results of the LAMP assay were compared with those of the PCR assay.

Sensitivity of LAMP and PCR assays. The five strains of TDH-producing *V. parahaemolyticus,* i.e., EKN5285 (serotype O3:K6 from sediment), EKN5301 (O4:K12 from a clinical specimen), EKN5302 (O4:K68 from a clinical specimen), EKN5731 (O3:K6 from a clinical specimen), and EKN5768 (O1:K1 from a clinical specimen), were tested for sensitivity in the LAMP and PCR assays. They were grown on heart infusion agar medium with 2.0% (wt/vol) NaCl at 37°C for 18 h. Colonies were suspended at a concentration of McFarland turbidity standard of 1 in 10 mM phosphate buffer (PB; pH 7.5) with 3% NaCl (3% NaCl-PB) and then serially 10-fold diluted to 10^{-4} to 10^{-7} in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 3% NaCl-PB. Each of the TE buffer–diluted series was denatured at 95°C for 5 min and centrifuged at 2,000 × *g* for 1 min. The supernatant was used as the template solution for LAMP and PCR assays in duplicate. In the 3% NaCl-PB diluted series, 100 μl of each dilution was plated onto heart infusion agar medium with 2% NaCl in duplicate. After incubation at 37°C for 18 h, the number of colonies was counted.

Detection of TDH-producing *V. parahaemolyticus* in artificially contaminated oyster samples. Fresh Japanese oysters (*Crassostrea gigas*) packed in polyethylene bags had been purchased from a retail market. These were transferred to our laboratories within 1 h and kept at 4°C for less than 6 h until they were used in the experiments. Approximately 100 g of Japanese oysters was homogenized by a blender in an ice bath for 1 min. The aerobic bacteria cells were enumerated by the spread plate technique on heart infusion agar medium with 2% NaCl. The homogenate was confirmed to be noncontaminated with TDH-producing *V. parahaemolyticus* by the LAMP and PCR assays described above. To each 25-g homogenized sample in stomach filter bags (Eiken Chemical Co. Ltd.) was added 225 ml of alkaline peptone water (pH 8.5). The colonies, grown on heart infusion agar with 2.0% (wt/vol) NaCl, of each TDH-producing *V. parahaemolyticus* (EKN5285) isolated from sediment, TDH-nonproducing *V. parahaemolyticus* (EKN5204) from a food sample, and *V. alginolyticus* (EKN5179) from a food sample were suspended in 2.5% NaCl at a concentration of McFarland turbidity standard of 1, which is approximately 3.0×10^8 CFU/ml. One of the homogenized oyster samples was inoculated with 0.1 ml of 10^{-6} dilutions of TDH-producing *V. parahaemolyticus,* 0.1 ml of 10^{-3} dilutions of TDH-nonproducing *V. parahaemolyticus,* and 0.1 ml of 10^{-2} dilutions of *V. alginolyticus.* The proportion of cell num-

TABLE 1. *Specificity of the LAMP assay for* tdh *gene*

Bacterial species	Serotype	No. of strains	Detection of *tdh* gene or TDH[a]			
			LAMP	PCR	RPLA	Kanagawa phenomenon
Vibrio parahaemolyticus	OUT:K5	1	−	−	−	NT
	OUT:K22	1	−	−	−	NT
	OUT:K29	1	−	−	−	−
	OUT:K32	1	−	−	−	−
	OUT:K57	1	−	−	−	−
	O1:KUT	1	−	−	−	−
	O1:K1	1	+	+	w	+
	O1:K25	1	+	+	+	+
	O1:K56	1	+	+	+	+
	O3:K6	19	+	+	+[b]	+
	O4:K9	2	+	+	+	+
	O4:K8	3	+	+	+	+
	O4:K12	1	+	+	+	+
	O4:K13	1	+	+	+	+
	O4:K49	1	−	−	−	−
	O4:K64	1	−	−	−	−
	O4:K68	2	+	+	+	+
	O10:K24	1	−	−	−	−
	O5:K68	1	+	+	+	+
	OUT:KUT	1	−	−	−	−
V. alginolyticus		3	−	−	NT	NT
V. anguillarum		1	−	−	NT	NT
V. cholerae		31	−	−	NT	NT
V. damsela		1	−	−	NT	NT
V. fluvialis		3	−	−	NT	NT
V. furnissii		1	−	−	NT	NT
V. metschnikovii		2	−	−	NT	NT
V. mimicus		5	−	−	NT	NT
V. pisoim var. *japonicus*		1	−	−	NT	NT
V. tryrogenes		1	−	−	NT	NT
V. vulnificus		2	−	−	NT	NT
Grimontia hollisae		1	+	+	−	+
Enterobacteriaceae						
Citrobacter freundii		2	−	−	NT	NT
C. koseri		1	−	−	NT	NT
Edwardsiella tarda		1	−	−	NT	NT
Enterobacter aerogenes		1	−	−	NT	NT
E. cloacae		1	−	−	NT	NT
Escherichia coli		1	−	−	NT	NT
E. fergusonii		1	−	−	NT	NT
E. hermannii		1	−	−	NT	NT
Hafnia alvei		1	−	−	NT	NT
Klebsiella oxytoca		1	−	−	NT	NT
K. pneumoniae		1	−	−	NT	NT
Morganella morganii		1	−	−	NT	NT
Proteus mirabilis		2	−	−	NT	NT
P. vulgaris		1	−	−	NT	NT
Providencia alcalifaciens		1	−	−	NT	NT
P. rettgeri		1	−	−	NT	NT
P. stuartii		1	−	−	NT	NT
Salmonella enterica		2	−	−	NT	NT
Serratia marcescens		3	−	−	NT	NT
Yersinia enterocolitica		1	−	−	NT	NT
Y. pseudotuberculosis		1	−	−	NT	NT
Pseudomonas aeruginosa		1	−	−	NT	NT
Gram-positive cocci						
Enterococcus avium		1	−	−	NT	NT
E. durans		1	−	−	NT	NT

TABLE 1. *Continued*

Bacterial species	Serotype	No. of strains	Detection of *tdh* gene or TDH[a]			
			LAMP	PCR	RPLA	Kanagawa phenomenon
Enterococcus faecalis		2	−	−	NT	NT
Lactobacillus lactis		1	−	−	NT	NT
Micrococcus luteus		2	−	−	NT	NT
Staphylococcus aureus		1	−	−	NT	NT
S. capitis		1	−	−	NT	NT
S. epidermidis		1	−	−	NT	NT
S. haemolyticus		1	−	−	NT	NT
S. intermedius		1	−	−	NT	NT
S. warneri		1	−	−	NT	NT
S. xylosus		1	−	−	NT	NT
Streptococcus pyogenes		1	−	−	NT	NT
Listeria monocytogenes		1	−	−	NT	NT

[a] +, positive reaction; w, weak reaction; −, negative reaction; NT, not tested.
[b] Two strains exhibited weak reaction.

bers of these strains was 1:1,000:10,000, respectively. Another sample was not inoculated with *V. parahaemolyticus* or *V. alginolyticus,* to be used as a negative control. The inoculated bacterial cells were enumerated by the spread plate technique on heart infusion agar medium with 2% NaCl. The artificially contaminated and noncontaminated samples were incubated at 37°C. Following enrichment for 0, 2, 3, 4, 6, and 18 h, the culture was filtered with a stomach filter bag. One milliliter of filtered culture was transferred to a microcentrifuge tube and centrifuged at 2,000 × *g* at room temperature for 1 min. The supernatant was removed, and then the precipitate was stored at −20°C within 24 h until DNA extraction. The genomic DNA of bacteria and oyster were extracted using an EXTRAGEN II Kit (TOSOH Co., Tokyo, Japan) with a modified protocol as follows. The precipitate was resuspended in 250 μl of TE buffer, 20 μl of Reagent I of the EXTRAGEN II kit was added, and then, 1,000 μl of Reagent II was added, sequentially. The mixture was centrifuged at 12,000 × *g* at 4°C for 3 min, and the supernatant was discarded. The pellet was resuspended in 1,000 μl of Reagent II and centrifuged at 12,000 × *g* at 4°C for 3 min, and then the supernatant was discarded. The pellet was resuspended in 1,000 μl of Reagent II and centrifuged at 12,000 × *g* at 4°C for 3 min, and then the supernatant was discarded. The pellet was resuspended in 300 μl of Reagent III and centrifuged at 12,000 × *g* for at 4°C for 3 min, and the supernatant was discarded. Finally, the pellet was rinsed with 70% ethanol and dissolved in 100 μl of TE buffer. The solution including the DNA was denatured at 95°C for 5 min and centrifuged at 2,000 × *g* for 1 min. The supernatant was used as the template for the LAMP and PCR assays. These experiments were carried out in duplicate.

RESULTS

In the 32 strains of TDH-producing *V. parahaemolyticus* and one strain of *G. hollisae,* significant amplification was observed in the LAMP and PCR assays. In 10 strains of TDH-nonproducing *V. parahaemolyticus* and 94 strains of bacteria, amplifications were not observed in the LAMP and PCR assays (Table 1). In the RPLA assay, 32 strains of TDH-producing *V. parahaemolyticus* exhibited a positive reaction, and 10 strains of TDH-nonproducing *V. parahaemolyticus* and one strain of *G. hollisae* exhibited a negative reaction. There were no differences among the results for most strains in RPLA, LAMP, and PCR assays and the Kanagawa phenomenon. However, *G. hollisae* was positive for the Kanagawa phenomenon and in the LAMP and PCR assays but not in the RPLA assay.

The detection limits of the LAMP assay in five strains were determined. The sensitivity of the LAMP assay was compared with that of the PCR assay (Table 2). In both the LAMP and PCR assays, the 10^{-6} dilutions of all strains were positive and the lowest number of cells in the test tubes at this dilution was 0.9 CFU.

Detection of TDH-producing *V. parahaemolyticus* in oysters by the LAMP assay was investigated with oysters artificially contaminated with TDH-producing *V. parahaemolyticus* (1.2 CFU/g), TDH-nonproducing *V. parahaemolyticus* (8.0×10^2 CFU/g) and *V. alginolyticus* (2.4×10^4 CFU/g). The number of aerobic bacteria in oysters was 7.0×10^3 CFU/g. After incubation for 0, 2, 3, 4, 6, and 18 h at 37°C, the culture was subjected to DNA extraction. The *tdh* gene was amplified by LAMP and PCR assays in duplicate (Fig. 2). Figure 2A shows the results of turbidity measurement for the LAMP reaction for 60 min at 65°C in the artificially contaminated sample. One of the duplicated assays in the 3-h incubation sample and both of the duplicated assays in the samples of >4-h incubation resulted in significant amplification. The *tdh* gene was not amplified in the sample not artificially contaminated. The white precipitate of magnesium pyrophosphate in tubes after LAMP reaction was visible in the 4-, 6-, and 18-h incubation samples but invisible in the 0-h incubation sample (Fig. 2B). Figure 2C shows the electrophoresis of the PCR products. In the samples of 0, 2, 3, and 4 h of incubation, many nonspecific bands appeared. Specific amplicons (251 bp) were found in the samples of 6 and 18 h of incubation. Since the specific amplicons were obscure in the 6-h incubation sample, it was difficult to make a precise determination.

DISCUSSION

In this study, a set of primers was designed for detecting the *tdh* gene, and this is the first report demonstrat-

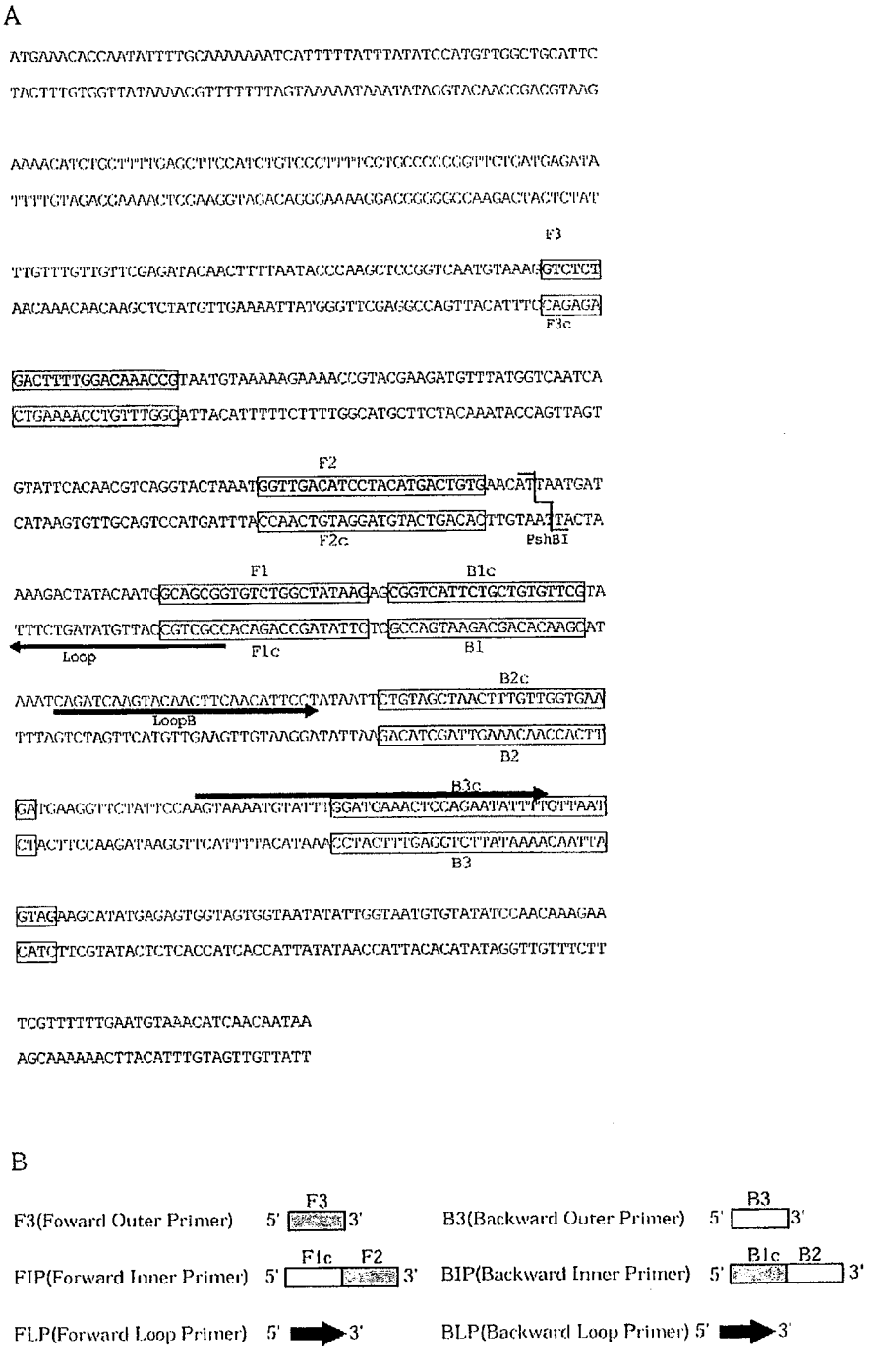

FIGURE 1. *(A) Nucleotide sequences of the* tdh *ORF (GenBank accession no. AY249144) used to design the six primers. The primer recognition regions of primers are shown by the boxes (inner and outer primers) and arrows (loop primers). F1c and B1c indicate the complementary sequences of F1 and B1, respectively. PshBI indicates the recognition site of the restriction enzyme* PshBI. *(B) Schematic diagram of the two outer (F3 and B3), two inner (FIP and BIP), and two loop (FLP and BLP) primers for LAMP.*

ing the usefulness of the LAMP assay to detect TDH-producing *V. parahaemolyticus.*

The specificity of the LAMP assay was the same as that of the PCR assay (Table 1), and the primer set designed in the LAMP assay has high specificity for detecting the *tdh* gene. Three strains of *V. parahaemolyticus* that exhibited a positive reaction in the LAMP and PCR assays had weak agglutination in the RPLA assay for TDH and were positive for Kanagawa phenomenon caused by TDH. On the other hand, one strain of *G. hollisae* that exhibited a positive reaction for the Kanagawa phenomenon was negative for the agglutination in the RPLA assay, although the results in the LAMP and PCR assays were positive. The LAMP and PCR assays might more clearly detect TDH-producing *V. parahaemolyticus* than does the RPLA assay. Tests using additional strains of TDH-producing *V. parahaemolyticus* should provide a more useful evaluation, although 32 strains were used in this study.

In the test for the sensitivity of LAMP and PCR assays, <1 CFU of TDH-producing *V. parahaemolyticus* in a test tube was detected (Table 2). The cultures of *V. parahaemolyticus* by incubation at 37°C for 18 h were used in the test. The cultures might be in stationary phase, including a high proportion of cells that do not produce CFU. However, the sensitivity was similar to the results of a test using log-phase culture by incubation for 5 h, which was performed in a preliminary study. Therefore, the test for the sensitivity of LAMP and PCR assays in this study was not affected

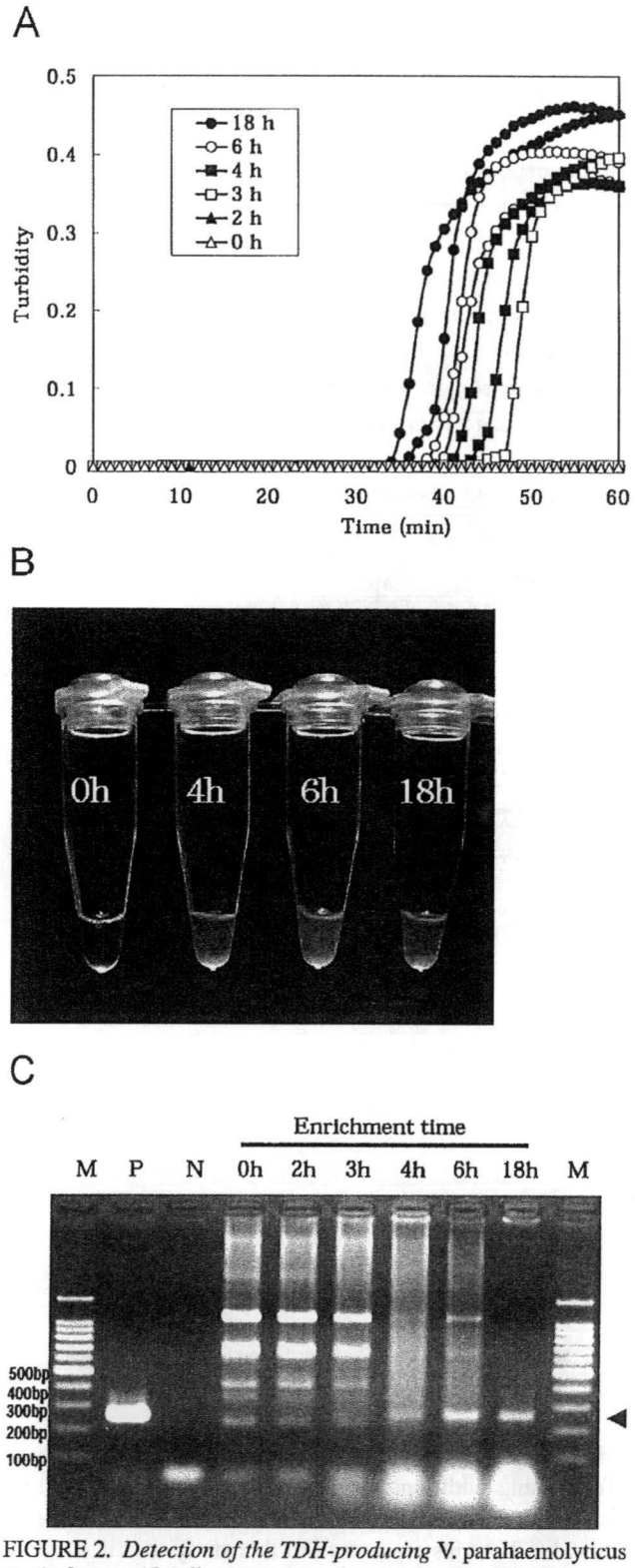

FIGURE 2. *Detection of the TDH-producing* V. parahaemolyticus *strain from artificially contaminated oyster samples. The Japanese oysters were inoculated with 1.2 CFU of TDH-producing* V. parahaemolyticus *(EKN5285) per g, 8.0 × 10² CFU of TDH-nonproducing* V. parahaemolyticus *(EKN5204) per g, and 2.4 × 10⁴ CFU of* V. alginolyticus *(EKN5179) per g. (A) Turbidity measurements of the LAMP reaction with LA320C real-time turbidimeter for LAMP. The enrichment culture of oysters in APW (alkaline peptone water) for 2, 3, 4, 6, and 18 h were tested in the LAMP assay in duplicate. (B) Reaction tubes after LAMP assay for 60 min. (C) Agarose gel electrophoresis of the PCR products. The specific amplicons (251 bp) are indicated by arrowheads. M, 100-bp DNA ladder marker (Takara).*

TABLE 2. *Sensitivity of the LAMP and PCR assays*

Serotype of *V. parahaemolyticus*/ strain	Dilution	No. of cells in tube	Reaction[a] (no. positive/total no.)	
			LAMP	PCR
O3:K6/EKN5285	10^{-4}	420	NT	+ (2/2)
	10^{-5}	42	+ (2/2)	+ (2/2)
	10^{-6}	4.2	+ (2/2)	+ (2/2)
	10^{-7}	0.4	+ (1/2)	− (0/2)
O4:K12/EKN5301	10^{-4}	420	+ (2/2)	+ (2/2)
	10^{-5}	42	+ (2/2)	+ (2/2)
	10^{-6}	4.2	+ (2/2)	+ (2/2)
	10^{-7}	0.4	− (0/2)	− (0/2)
O4:K68/EKN5302	10^{-4}	260	+ (2/2)	+ (2/2)
	10^{-5}	26	+ (2/2)	+ (2/2)
	10^{-6}	2.6	+ (2/2)	+ (2/2)
	10^{-7}	0.3	+ (1/2)	+ (1/2)
O3:K6/EKN5731	10^{-4}	140	+ (2/2)	+ (2/2)
	10^{-5}	14	+ (2/2)	+ (2/2)
	10^{-6}	1.4	+ (2/2)	+ (2/2)
	10^{-7}	0.1	− (0/2)	+ (2/2)
O1:K1/EKN5768	10^{-4}	90	+ (2/2)	+ (2/2)
	10^{-5}	9	+ (2/2)	+ (2/2)
	10^{-6}	0.9	+ (2/2)	+ (2/2)
	10^{-7}	0.1	+ (1/2)	+ (1/2)

[a] +, positive reaction; −, negative reaction.

by cells that do not produce CFU. Nishibuchi and Kaper *(15)* reported that duplication and variation of the *tdh* gene are encoded in a chromosome of *V. parahaemolyticus*. The LAMP and PCR assays in this study were designed to detect both *tdh1* and *tdh2* genes. Thus, detecting <1 CFU of TDH-producing *V. parahaemolyticus* should be due to the duplication and variation of *tdh* gene.

It is difficult to isolate TDH-producing *V. parahaemolyticus* from seafood because a large number of TDH-nonproducing *Vibrio* spp. were contaminated with a small number of TDH-producing *V. parahaemolyticus* organisms *(11)*. It is expected that oysters are often contaminated with high populations of halophilic bacteria such as TDH-nonproducing *V. parahaemolyticus* and *V. alginolyticus*. Therefore, we investigated the sensitive detection by LAMP assay in oyster samples inoculated with these bacteria. In this study, a period of 6 h was required to detect TDH-producing *V. parahaemolyticus* in oysters, using a combination of enrichment for 4 h, DNA extraction for 1 h, and the LAMP assay for 1 h. It is noteworthy that TDH-producing *V. parahaemolyticus* in oysters were detected within a period of only 1 day by using this assay.

One of the unique characteristics of LAMP is the capacity it affords for visual judgment of nucleic acid amplification. Mori et al. *(12)* originally reported that amplification by this assay can be detected by confirming the presence of magnesium pyrophosphate, a white precipitate generated as a by-product during the reaction. Some researchers have reported that detecting a small amount of the white precipitate by observation with the naked eye is not always easy because the negative-reaction mixture has

white precipitate due to template-related obstacles such as proteins in the sample and the components of the culture medium. In this study, obstacles were removed from the solution of DNA template to make clear a negative reaction (Fig. 2B). The positive reactions in samples subjected to 4, 6, and 18 h of incubation were sharply contrasted to the negative reaction in 0-h incubation samples (Fig. 2B). In an analysis of PCR products by agarose gel electrophoresis, a specific amplicon (251 bp) was detectable in 6-h incubation but not 4-h incubation. The total time required from enrichment to detection was more than 10 h: enrichment required more than 6 h, DNA extraction required 1 h, PCR assay required 2 h, and electrophoresis required 1 h. Therefore, the PCR assay is not valuable to detect TDH-producing *V. parahaemolyticus* in oysters within a period limited to 1 day. Real-time PCR was much faster than PCR: it was possible to detect TDH-producing *V. parahaemolyticus* by using the sample preparation and real-time PCR within 1 day *(18)*; however, real-time PCR requires sophisticated equipment. LAMP amplification is performed under isothermal conditions, between 60 and 65°C *(8, 13, 19)*, using simple incubators such as a water bath or block heater, which is sufficient for DNA amplification. Visual judgment eliminates the need for special procedures postamplification such as electrophoresis.

This study demonstrates that the LAMP assay is effective in rapidly detecting TDH-producing *V. parahaemolyticus* with high sensitivity in seafood, and the assay has the capacity for use in the field or in the small-scale laboratory.

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