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Development of a Loop-Mediated Isothermal Amplification Assay for Sensitive and Rapid Detection of the *tdh* and *trh* Genes of *Vibrio parahaemolyticus* and Related *Vibrio* Species[▽]

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Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are the major virulence determinants of *Vibrio parahaemolyticus*. TRH is further differentiated into TRH1 and TRH2 on the basis of genetic and phenotypic differences. We developed a novel and highly specific loop-mediated isothermal amplification (LAMP) assay for sensitive and rapid detection of the *tdh*, *trh*1, and *trh*2 genes of *V. parahaemolyticus*. The LAMP assay was designed for both combined and individual detection of the *tdh*, *trh*1, and *trh*2 genes and combined detection of the *trh*1 and *trh*2 genes. Our results showed that it gave the same results as DNA probes and conventional PCR assays for 125 strains of *V. parahaemolyticus*, 3 strains of *Grimontia hollisae*, and 2 strains of *Vibrio mimicus* carrying the *tdh*, *trh*1, and *trh*2 genes in various combinations. No LAMP products were detected for any of the 20 bacterial strains lacking the *tdh*, *trh*1, and *trh*2 genes. The sensitivities of the LAMP assay for detection of *tdh*-, *trh*1-, and *trh*2-carrying *V. parahaemolyticus* strains in spiked shrimp samples were 0.8, 21.3, and 5.0 CFU per LAMP reaction tube, respectively. Starting with DNA extraction from a single colony and from spiked shrimp samples, the LAMP assay required only 27 to 60 min and less than 80 min, respectively. This is the first report of a rapid and specific LAMP assay for detection and differentiation of the *tdh*, *trh*1, and *trh*2 genes of *V. parahaemolyticus* and related *Vibrio* species.

Vibrio parahaemolyticus, which is widely distributed in estuarine, marine, and coastal environments of tropical and temperate zones, causes seafood-borne gastrointestinal disorders in humans (9). Because most clinical isolates of *V. parahaemolyticus* produce the thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), or both (5, 11, 14), these products are considered important virulence markers of *V. parahaemolyticus* (4, 5, 9, 11, 14). TDH and TRH are encoded by the *tdh* and *trh* genes, respectively. Five sequence variants of the *tdh* gene (*tdh*1 to *tdh*5) can be distinguished, which are >97% identical (1, 10). The *tdh* gene has also been detected in *Grimontia* (*Vibrio*) *hollisae* and some strains of *Vibrio mimicus* isolated from patients with diarrhea (9). The *trh* gene shares ca. 68% sequence identity with the *tdh* gene (5). Although *trh* gene sequences vary somewhat among strains, the *trh* variants can be clustered into two subgroups represented by two *trh* genes (*trh*1 and *trh*2), which share 84% sequence identity (5).

Although most clinical isolates carry the *tdh* and *trh* genes, either alone or in combination, approximately 99% of environmental isolates do not possess either gene (9). These genes are therefore considered important virulence and epidemiological markers (5, 11, 14). Detection of the *tdh* and *trh* genes of *V. parahaemolyticus* using DNA probe methods is time-consuming and laborious. PCR assays, in contrast, although providing rapid detection of both *tdh* and *trh* genes (2, 15), require electrophoresis on an agarose gel, which is time-consuming and tedious. A recent real-time PCR assay for detection of the *tdh* and *trh* genes (12) is more rapid than conventional PCR assays but requires sophisticated and expensive equipment.

A recently developed novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) (13) is a promising candidate for rapid and easy detection of the *tdh* and *trh* genes. A LAMP assay allows one-step detection of gene amplification by simple turbidity analysis and requires only a simple incubator, such as a heat block or a water bath providing a constant temperature. LAMP assays are faster, easier to perform, and more specific than conventional PCR assays (6, 7). Further, they synthesize a large amount of DNA and its by-product, an insoluble white precipitate of magnesium pyrophosphate, and the by-product can be detected by simple turbidity analysis. The increase in the turbidity of the reaction mixture due to the production of the white precipitate correlates with the amount of DNA synthesized (6, 7, 13). Thus, LAMP assays do not require expensive equipment and are highly precise (3, 18, 19).

Here we describe a rapid and simple LAMP assay for detection of the *tdh*, *trh*1, and *trh*2 genes of *V. parahaemolyticus*. We also determined the sensitivity of this LAMP assay using spiked shrimp samples.

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MATERIALS AND METHODS

Bacterial strains. Bacterial strains belonging to *V. parahaemolyticus*, other *Vibrio* species, and *Grimontia* (*Vibrio*) *hollisae* were used. All cultures were stock

TABLE 1. LAMP primers used in this study[a]

Primer	Sequence (5′ to 3′)	Gene location
Tdh-FIP	GTACCTGACGTTGTGAATACTGATTGTCTCTGACTTTTGGACAAAC	353–327, 268–288
Tdh-BIP	TGACATCCTACATGACTGTGAACACTTATAGCCAGACACCGC	362–385, 429–412
Tdh-F3	AGATATTGTTTGTTGTTCGAGAT	209–231
Tdh-B3	AACACAGCAGAATGACCG	449–432
Tdh-LF	GTACGGTTTTCTTTTTACATTACG	312–289
Tdh-LB	AAGACTATACAATGGCAGCG	395–414
Trh1-FIP	AGGCTTGTTTTTTCTGATTTTGTGACTACACAATGGCTGCTCT	418–394, 343–360
Trh1-BIP	TCTTCTGTTAGTGATTTCGTTGGTTTTCATCCAAATACGTTACACT	432–455, 498–477
Trh1-F3	GCGCCTATATGACGGTAA	310–327
Trh1-B3	ACATTGACGAAATATTCTGGC	520–500
Trh1-LF	AGACCGTTGARAGGCC	393–378
Trh2-FIP	CCGATTGACCGTATACATCTTTGTTGTGGAGGACTATTGGACAA	493–470, 426–445
Trh2-BIP	TCAAAGTGGTTAAGCGCCTATATGCCATSTTTATAACCAGAAAGAGC	511–534, 593–571
Trh2-F3	CATCAATACCTTTTCCTTCTCC	335–356
Trh2-B3	GCTTGTTTTCTCTGATTTTGTG	630–609
Trh2-LF	TGGTTTTCTTTTTATGKTTCGGT	468–446
Trh2-LB	ATGGTCAYAACTATACRATGGC	548–569

[a] All primers were designed using the sequence of the *tdh* gene of *V. parahaemolyticus* X54340, the *trh1* gene of S67850, and the *trh2* gene of M88112 deposited in the GenBank database.

cultures from our laboratory which were originally isolated at locations throughout the world over a 30-year period.

Detection of virulence genes. To detect the *tdh* and *trh* genes, a PCR assay was carried out using primers D3 (5′-CCACTACCACTCTCATATGC-3′) and D5 (5′-GGTACTAAATGGCTGACATC-3′) and primers R2 (5′-GGCTCAAAAT GGTTAAGCG-3′) and R6 (5′-CATTTCCGCTCTCATATGC-3′), respectively, as previously described (15). *trh*-positive PCR samples were examined further to determine the presence of the *trh*1 and *trh*2 genes by performing a DNA colony hybridization assay as described previously (5, 11). In brief, the DNA probes specific for the *trh*1 and *trh*2 genes were 334- and 419-bp DNA fragments, respectively, isolated from recombinant plasmids and were labeled by the random priming method with ^{32}P-labeled dCTP. DNA colony blots were prepared on nitrocellulose membranes, and hybridization was performed under high-stringency conditions (in a solution containing 50% formamide). Hybridization signals on X-ray film were judged visually. Colony blots giving strong and weak hybridization signals with the *trh*1 and *trh*2 probes, respectively, were considered positive for the *trh*1 gene, while colony blots that gave weak and strong hybridization signals with the *trh*1 and *trh*2 probes, respectively, were considered positive for the *trh*2 gene.

Extraction of DNA from pure bacterial cultures for the LAMP assay. Bacterial DNA for the LAMP assay was extracted as described previously (18, 19). In brief, a loopful (1 μl) of bacterial growth on an appropriate agar medium was inoculated into 50 μl of an NaOH solution (25 mmol liter^{-1}) in a 1.5-ml microcentrifuge tube using a disposable inoculating loop (diameter, ca. 1 mm; Nunc Ltd., Roskilde, Denmark), and the cell mixture was heated at 95°C for 5 min. After neutralization with 4 μl of Tris-HCl buffer (1 mol liter^{-1}, pH 7.5), cell debris was pelleted by centrifugation at 20,000 × *g* and 4°C for 5 min, and the supernatant was used as the template DNA for the LAMP assay.

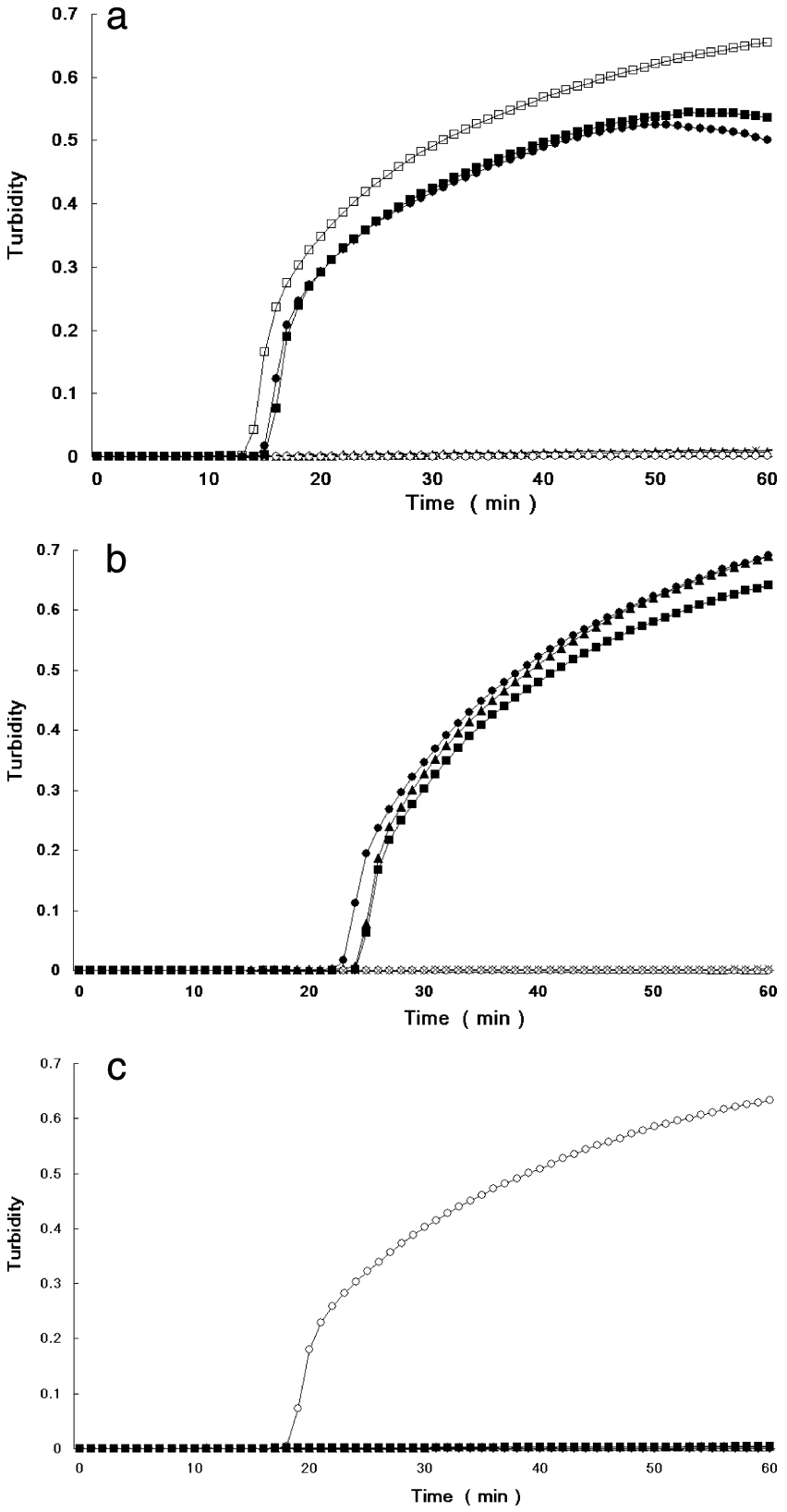
Primer design for the LAMP assay. All primers were designed using Primer Explorer V4 software (Fujitsu System Solutions Ltd., Tokyo, Japan) and were synthesized using sequence-grade purification by Hokkaido System Science Co., Ltd. (Sapporo, Japan). Nucleotide sequences specific for the *tdh*, *trh*1, and *trh*2 genes were detected by multiple alignments of 22 *tdh* sequences, 6 *trh*1 sequences, and 12 *trh*2 sequences available from the DDBJ/EMBL/GenBank database. The sequences selected were used as LAMP primers (Table 1).

LAMP assay. The LAMP assay was performed using a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan) as previously described (18, 19). Individual LAMP assays, which targeted the *tdh*, *trh*1, and *trh*2 genes individually, were performed using 25-μl 1× reaction mixtures (Eiken Chemical) containing 1 μl of template DNA, 1 μl of *Bst* DNA polymerase (Eiken Chemical), 1.6 μmol liter^{-1} each of inner primers FIP and BIP, 0.2 μmol liter^{-1} each of outer primers F3 and B3, and 0.8 μmol liter^{-1} each of loop primers LF and LB. The reaction mixtures were incubated in a Loopamp real-time turbidimeter (LA-320; Teramecs Co. Ltd., Kyoto, Japan) at 65°C for 60 min and then at 80°C

TABLE 2. Results of LAMP assays for detection of the *tdh*, *trh*1, and *trh*2 genes

Species	Genotype[a]	No. of strains tested	No. of LAMP-positive strains using a primer set(s) for:				
			tdh	*trh*1	*trh*2	*tdh*, *trh*1, and *trh*2	*trh*1 and *trh*2
V. parahaemolyticus	*tdh*$^+$, *trh* negative	30	30	0	0	30	0
V. parahaemolyticus	*tdh*$^+$, *trh*1$^+$	32	32	32	0	32	32
V. parahaemolyticus	*tdh* negative, *trh*1$^+$	13	0	13	0	13	13
V. parahaemolyticus	*tdh*$^+$, *trh*2$^+$	13	13	0	13	13	13
V. parahaemolyticus	*tdh* negative, *trh*2$^+$	37	0	0	37	37	37
V. parahaemolyticus	*tdh* negative, *trh* negative	10	0	0	0	0	0
G. hollisae	*tdh*$^+$, *trh* negative	3	3	0	0	3	0
V. mimicus	*tdh*$^+$, *trh* negative	2	2	0	0	2	0
V. alginolyticus	*tdh* negative, *trh* negative	3	0	0	0	0	0
V. cholerae	*tdh* negative, *trh* negative	1	0	0	0	0	0
V. fluvialis	*tdh* negative, *trh* negative	2	0	0	0	0	0
V. furnissii	*tdh* negative, *trh* negative	1	0	0	0	0	0
V. metschnikovii	*tdh* negative, *trh* negative	1	0	0	0	0	0
V. mimicus	*tdh* negative, *trh* negative	1	0	0	0	0	0
V. vulnificus	*tdh* negative, *trh* negative	1	0	0	0	0	0

[a] Determined by PCR and DNA colony hybridization assays.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Turbidity
Time (min)
b
Turbidity
Time (min)
c
Turbidity
Time (min)

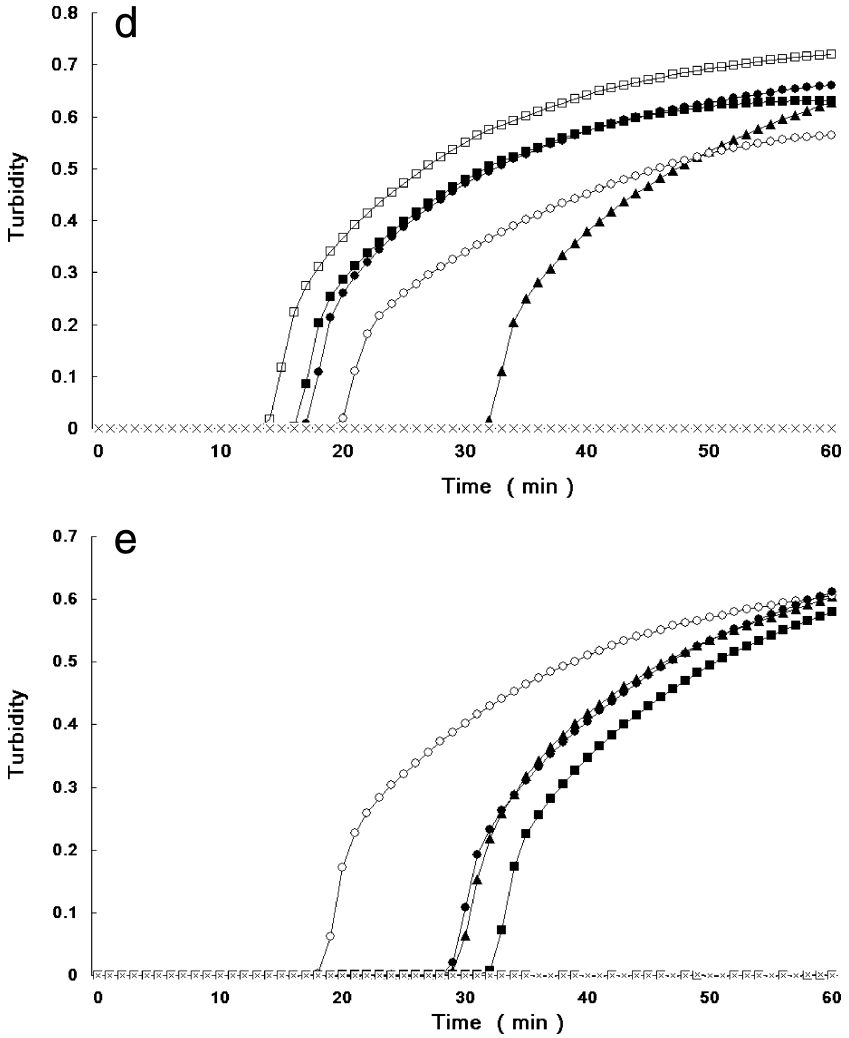


FIG. 1. Real-time monitoring of LAMP reactions with *V. parahaemolyticus* control strains for multiplex and individual detection of the *tdh*, *trh*1, and *trh*2 genes. Profiles for individual detection of *tdh* (a), *trh*1 (b), and *trh*2 (c) and for multiplex detection of *tdh*, *trh*1, and *trh*2 (d) and of *trh*1 and *trh*2 (e) are shown. Total DNAs extracted from AQ3815 (*tdh*1$^+$, *tdh*2$^+$, *trh*1 negative, *trh*2 negative) (□), AQ3776 (*tdh*3$^+$, *tdh*4$^+$, *trh*1$^+$, *trh*2 negative) (■), AQ3860 (*tdh*5$^+$, *trh*1$^+$, *trh*2 negative) (●), AQ4037 (*tdh* negative, *trh*1$^+$, *trh*2 negative) (▲), AT4 (*tdh* negative, *trh*1 negative, *trh*2$^+$) (○), and Vp02 (*tdh* negative, *trh*1 negative, *trh*2 negative [negative control]) (×) were examined.

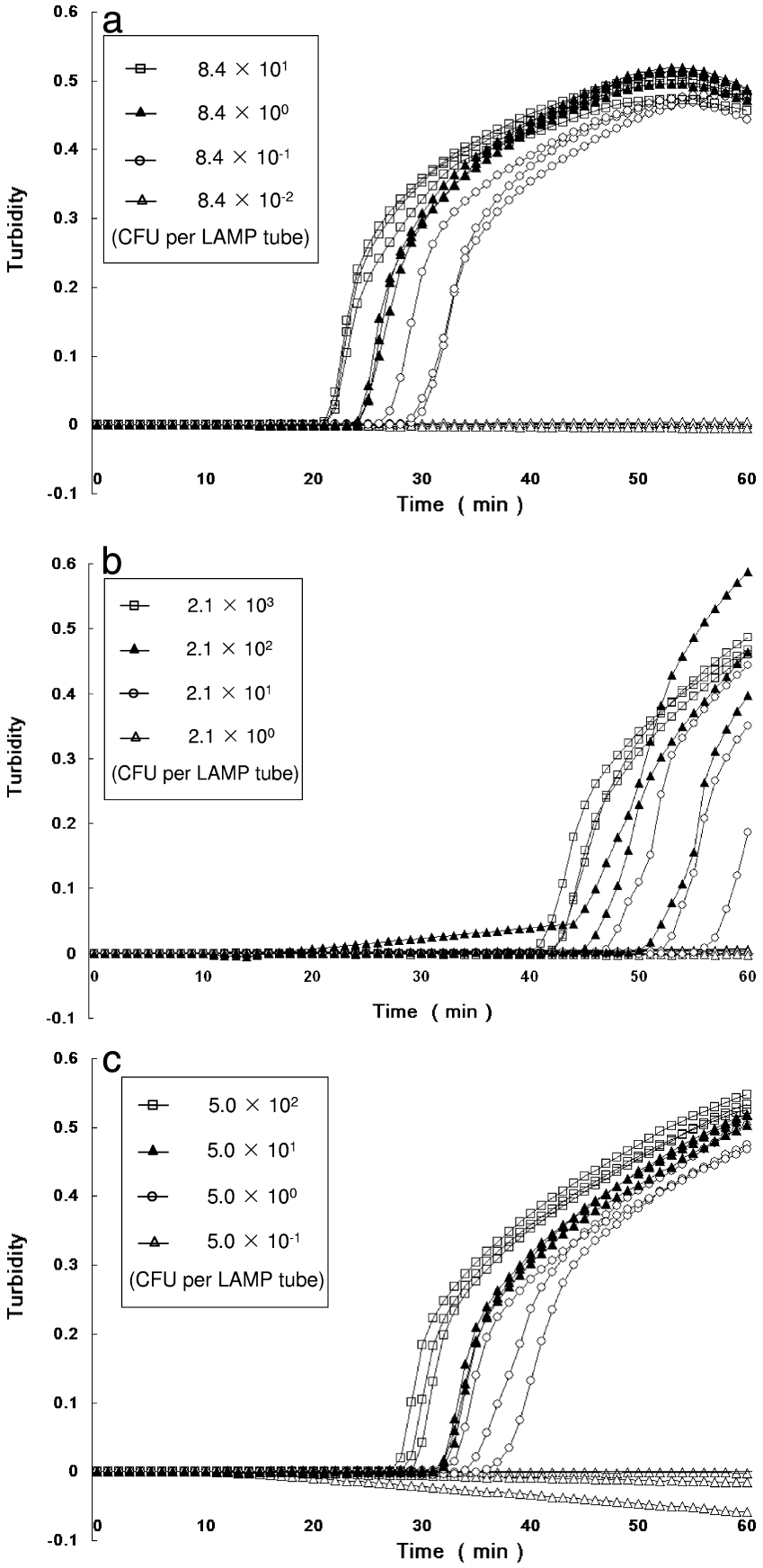
for 2 min to complete the reaction. LAMP was detected in real time by determining the turbidity at 650 nm using an LA-320 turbidimeter. A reaction was considered positive when the turbidity reached 0.1 within 60 min. White precipitation visible with the unaided eye was also considered an indication of a successful LAMP procedure (6).

LAMP assays using a mixture of the *tdh*, *trh*1, and *trh*2 primer sets and a mixture of the *trh*1 and *trh*2 primer sets were also performed to evaluate if multiplex assays were possible under the same conditions.

Sensitivity of the LAMP assay with spiked shrimp samples. (i) Preparation of shrimp samples. The sensitivity of the LAMP assay with spiked shrimp (*Trachypenaeus curvirostris*) samples was determined as previously described (17, 18), with slight modifications. Shrimp samples were purchased at a supermarket in Osaka, Japan, in 2009, and whole bodies were used without peeling the shells. For preparation of the template DNA, 25 g of a shrimp sample was added to 225 ml of alkaline peptone water (APW) (Eiken Chemical) in a plastic stomacher bag and stomached for 30 s. After overnight incubation at 36°C, APW enrichment cultures were confirmed to be *V. parahaemolyticus* negative by plating onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar and examination of the APW enrichment cultures using a *V. parahaemolyticus* species-specific LAMP assay (18). *V. parahaemolyticus*-negative APW enrichment cultures were spiked with positive and negative control strains for the sensitivity test.

(ii) Preparation of bacterial cells. In parallel, a small amount of bacterial growth on TCBS agar was inoculated into 4 ml of tryptic soy broth (TSB) (Becton Dickinson) and incubated overnight at 36°C. Then 40 μl of an enriched TSB culture was transferred to 4 ml of TSB and incubated for 4 to 5 h with shaking at 150 rpm at 36°C in a water bath to obtain mid-log-phase cells. Serial 10-fold dilutions of the test strain cultures were prepared in phosphate-buffered saline (pH 7.5).

(iii) Preparation of template DNA. In a parallel experiment, a 100-μl mid-log-phase APW culture of a negative control strain, *V. parahaemolyticus* VP02 (*tdh* negative, *trh* negative; 5.7×10^7 CFU/ml), and 100 μl each of serial dilutions of positive control strains in the mid-log phase were inoculated into 900 μl of APW enrichment broth in 1.5-ml microcentrifuge tubes. After thoroughly mixed using a Vortex-Genie 2 (Scientific Industries Inc., NY), the spiked samples were centrifuged at 900 × *g* for 1 min to remove larger debris. Each resulting supernatant was transferred to a new 1.5-ml microcentrifuge tube and centrifuged at 10,000 × *g* for 5 min. After removal of the supernatant, the pellets were resuspended in 100 μl of NaOH (25 mmol liter^{-1}), thoroughly mixed using a Vortex-Genie 2, and then heated at 95°C for 5 min. After neutralization with 8 μl of Tris-HCl buffer (1 mol liter^{-1}, pH 7.5), debris was removed by centrifugation at 20,000 × *g* at 4°C for 5 min. Supernatants were collected, and 1- or 2-μl portions were used as the template DNAs for LAMP assay.


a
8.4 × 10^1
8.4 × 10^0
8.4 × 10^-1
8.4 × 10^-2
(CFU per LAMP tube)
Turbidity
Time (min)
b
2.1 × 10^3
2.1 × 10^2
2.1 × 10^1
2.1 × 10^0
(CFU per LAMP tube)
Turbidity
Time (min)
c
5.0 × 10^2
5.0 × 10^1
5.0 × 10^0
5.0 × 10^-1
(CFU per LAMP tube)
Turbidity
Time (min)

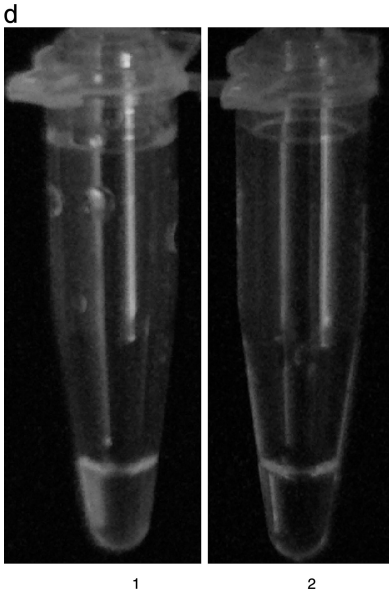


FIG. 2. Sensitivity test for detection of the *tdh*, *trh*1, *trh*2 genes in *V. parahaemolyticus* strains in spiked shrimp samples by real-time turbidimetry. The curves from left to right indicate decreasing concentrations of CFU from bacterial colonies. (a) Detection of the *tdh* gene of *V. parahaemolyticus* AQ3815. (b) Detection of the *trh*1 gene of *V. parahaemolyticus* AQ4037. (c) Detection of the *trh*2 gene of *V. parahaemolyticus* AT4. (d) Visual detection of the *tdh* gene by observation of precipitation. Tube 1, 84.4 CFU of AQ3815; tube 2, 0.1 CFU of AQ3815.

(iv) Determination of sensitivity. Using the template DNAs prepared as described above, the sensitivities of the individual LAMP assays for the *tdh*, *trh*1, and *trh*2 genes were determined in triplicate, and the detection limits were defined as the highest dilutions at which all three samples tested positive. The sensitivities of the multiplex LAMP assays (combined detection of the *tdh*, *trh*1, and *trh*2 genes and combined detection of the *trh*1 and *trh*2 genes) were also determined as described above, except that primers for the three and two genes were used simultaneously. In parallel, to determine the inoculum size, 100 μl of each dilution was plated onto tryptic soy agar (TSA) (Nissui, Tokyo, Japan) supplemented with 2.5% NaCl in duplicate and incubated overnight at 36°C. Further, the sensitivity of the LAMP assay for spiked APW enrichment cultures with different ratios of the bacteria (AQ3815/AQ4037/AT4/VP02 ratios of 1:1:1:1, 2:2:2:1, 4:4:4:1, and 8:8:8:1, which corresponded to CFU ratios of 0.8:21.3:5.0:105,000, 1.7:42.6:10.0:105,000, 3.4:85.2:20.0:105,000, and 6.7:170.4:40.0:105,000, respectively, in LAMP reaction tubes) was determined.

RESULTS

LAMP products were detected for all 125 strains of *V. parahaemolyticus*, 3 strains of *G. hollisae*, and 2 strains of *V. mimicus* carrying the *tdh*, *trh*1, and *trh*2 genes in various combinations (Table 2). No LAMP products were detected for any of the 20 bacterial strains not carrying the *tdh*, *trh*1, and *trh*2 genes. These results were perfectly consistent with those obtained by PCR and DNA colony hybridization assays.

The PCR assay took more than 4 h, but the LAMP assay using DNA extracted from a single colony on TCBS agar was markedly faster, requiring only 14 to 22 min for amplification of *tdh*, 24 to 38 min for amplification of *trh*1, and 18 to 28 min for amplification of *trh*2. Further, the multiplex LAMP assay for comprehensive detection of the hemolysin genes required 14 to 36 min for amplification of *tdh*, *trh*1 and *trh*2 and 20 to 47 min for amplification of *trh*1 and *trh*2. Representative results for the individual *tdh*, *trh*1, and *trh*2 LAMP assays obtained using an LA-320 turbidimeter are shown in Fig. 1a, 1b, and 1c, respectively. Results for combined detection of *tdh*, *trh*1, and *trh*2 and for combined detection of *trh*1 and *trh*2 are shown in Fig. 1d and 1e, respectively. When *tdh*-bearing *G. hollisae* and *V. mimicus* strains were used, the times for the LAMP assay were 20 to 23 min and 16 to 18 min, respectively, for *tdh* detection and 24 to 25 min and 16 to 18 min, respectively, for *tdh* detection by the multiplex (*tdh*, *trh*1, and *trh*2) system using DNA extracted from a single colony on TCBS agar or TSA (data not shown). All LAMP detection assays, including the DNA extraction step, were completed in 27 to 60 min. An increase in turbidity due to the LAMP reaction was confirmed both by using the LA-320 turbidimeter and by visual inspection, and the results of the two methods agreed perfectly.

As shown in Fig. 2 and Table 3, the sensitivities of the individual LAMP assays for *V. parahaemolyticus* AQ3815 (*tdh*$^+$, *trh* negative), AQ4037 (*tdh* negative, *trh*1$^+$) and AT4 (*tdh* negative, *trh*2$^+$) in spiked shrimp samples were 0.8, 21.3, and 5.0 CFU per LAMP tube, respectively. Dilutions yielding 84.4 to 0.8 CFU per LAMP tube (Fig. 2d, tube 1), 2,130 to 21.3 CFU per LAMP tube, and 500 to 5.0 CFU per LAMP tube showed an increase in turbidity, whereas dilutions yielding 0.1 CFU per LAMP tube (Fig. 2d, tube 2), 2.1 CFU per LAMP tube, and 0.5 CFU per LAMP tube did not show an increase. DNA amplification in the LAMP assay could also be judged using the white precipitate by visual assessment with the unaided eye. Representative LAMP results used for visual assessment with the unaided eye are shown in Fig. 2d. The sensitivities of the two methods were the same.

When sensitivity tests with shrimp inoculated with known amounts of positive control strains (AQ3815 [*tdh*$^+$], AQ4037 [*trh*1$^+$], and AT4 [*trh*2$^+$]) were performed using the two multiplex LAMP assays with mixtures of the *tdh*, *trh*1, and *trh*2 primer sets and the *trh*1 and *trh*2 primer sets, the sensitivities were slightly lower than those obtained with the LAMP assay

TABLE 3. Sensitivities of the LAMP assays for multiplex and individual detection of the *tdh*, *trh*1, and *trh*2 genes

Strain	Genotype	Sensitivity of the LAMP assay (CFU/LAMP tube) using a primer set(s) for[a]:				
		tdh	*trh*1	*trh*2	*tdh*, *trh*1, and *trh*2	*trh*1 and *trh*2
AQ3815	*tdh*$^+$	0.8	ND[b]	ND	1.7	ND
AQ4037	*trh*1$^+$	ND	21.3	ND	85.2	21.3
AT4	*trh*2$^+$	ND	ND	5.0	5.0	5.0

[a] The values are the lower limits of the number of viable cells detectable by the LAMP assay.
[b] ND, not determined.

TABLE 4. Sensitivity of the LAMP assay using DNA template mixtures obtained from small amounts of bacterial cells

No. of bacterial cells (CFU/LAMP tube)				LAMP results (no. positive/no. tested) using a primer set(s) for[a]:				
AQ3815	AQ4037	AT4	Vp02	*tdh*	*trh*1	*trh*2	*tdh*, *trh*1, and *trh*2	*trh*1 and *trh*2
6.7	170.4	40.0	105,000	ND	+ (3/3)	ND	ND	ND
3.4	85.2	20.0	105,000	ND	± (2/3)	ND	ND	ND
1.7	42.6	10.0	105,000	+ (3/3)	± (2/3)	+ (3/3)	ND	ND
0.8	21.3	5.0	105,000	± (2/3)	− (0/3)	± (2/3)	+ (3/3)	+ (3/3)

[a] +, triplicate assays were all positive. ±, triplicate assays were both positive and negative; −, triplicate assays were all negative; ND, not determined.

for individual gene detection (Table 3). Further, when sensitivity tests were carried out using shrimp samples inoculated with various amounts of the positive control strains and Vp02, a negative control strain, the two multiplex LAMP assays exhibited slightly higher sensitivities than the LAMP assays for individual gene detection (Table 4). In these sensitivity tests, all of the results obtained with the two multiplex LAMP assays were in agreement (Table 4).

DISCUSSION

Here we report a novel and highly specific LAMP assay for detection of the *tdh*, *trh*1, and *trh*2 genes. This assay provides markedly simpler and more rapid detection of the *tdh*, *trh*1, and *trh*2 genes than conventional PCR and DNA colony hybridization assays.

While our study was in progress, Nemoto and colleagues described a *tdh* LAMP assay performed with 42 strains of *V. parahaemolyticus* (8). However, this assay did not include a system to detect the *trh*1 and *trh*2 genes, which are also important virulence factors of *V. parahaemolyticus*. In contrast, our LAMP assay detects and differentiates the *tdh*, *trh*1, and *trh*2 genes and was evaluated using 135 strains of *V. parahaemolyticus* carrying these genes in various combinations. These strains represent isolates obtained from clinical cases in various locations around the world collected over a 30-year period. These assays should therefore prove to be useful for facilitating clinical diagnosis and for epidemiological investigations of outbreaks of food-borne *V. parahaemolyticus* infection.

Figure 3 shows a scheme for a suggested practical application of our LAMP system. The multiplex LAMP system, which allows detection of any of the *tdh*, *trh*1, and *trh*2 genes, is first system used to distinguish virulent and avirulent strains. Virulent strains are then distinguished into groups of strains with the *tdh* and *trh* genes by the *tdh*-specific and *trh*-specific LAMP assays, and the latter group is subsequently separated into *trh*1 and *trh*2 subtypes by the *trh*1- and *trh*2-specific LAMP assays.

All strains of *G. hollisae* and some strains of *V. mimicus* and *V. cholerae* isolated from patients with diarrhea are known to possess the *tdh* gene (9). Three *G. hollisae* and two *V. mimicus* *tdh*-carrying strains examined in this study were positive for the *tdh* gene as determined by our individual *tdh* LAMP assay. If this LAMP assay and the *V. parahaemolyticus* species-specific LAMP assay that we described previously (18) are combined, *tdh*-bearing non-*V. parahaemolyticus* species can be identified quickly and easily.

In our preliminary tests, at least 10 primer sets each were designed for the *tdh*, *trh*1, and *trh*2 genes and were evaluated for their specificity and speed of amplification (data not shown). The best sets were then selected (Table 1) and proven to be highly successful for the individual LAMP systems, as well as both of the multiplex LAMP systems (Tables 2, 3, and 4 and Fig. 2). One of the preliminary primer sets for the *trh*1 gene required only 14 to 25 min for amplification (data not shown). When a preliminary set of mixed primers comprising the *trh*1 primers, as well as the *tdh* and *trh*2 primers, that was finally adopted in the present study was used, the amplification time was conspicuously extended (35 to 70 min), and this set was thus considered unsuitable for rapid LAMP system detection. A possible explanation for this extension of the amplification time was the influence of competitive overlapping of the three primer sets in the coding region. The coding regions of the *tdh*, *trh*1, and *trh*2 genes are 567 bp long and have high

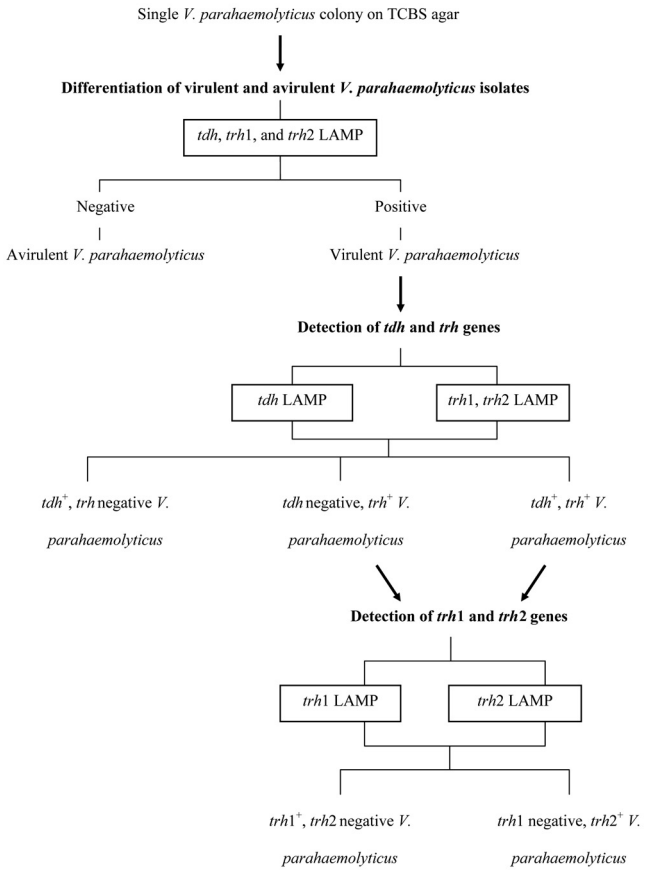


FIG. 3. Schematic representation of detection and differentiation of the *tdh*, *trh*1, and *trh*2 genes from a single *V. parahaemolyticus* colony on TCBS agar.

levels of sequence identity (around 68% for the *tdh* and *trh* genes and 84% for the *trh*1 and *trh*2 genes) (5). A *trh*1 primer set designed with another region was used as a second choice. We attempted to design a Trh1-LB primer for the sequence between two segments of the newly designed Trh1-BIP primer, corresponding to the gene location between positions 456 and 476 in S67850. However, a Trh1-LB primer could not be designed due to the insufficient G+C content of the region that the primer was intended to locate. This in turn decreased the sensitivity of the *trh*1 LAMP assay due to the lack of an LB primer to accelerate the LAMP reaction. As representative results in Fig. 1d show, comprehensive detection of the *tdh*, *trh*1, and *trh*2 genes was obtained in 14 to 36 min using the combination of the three primer sets, and therefore, we used these primer sets in the final assay.

The sensitivity of the *tdh* LAMP assay with spiked shrimp samples shown in Table 3 and Fig. 2 (0.8 CFU per LAMP reaction tube in triplicate) may appear to be somewhat high. However, previous studies have demonstrated that optimized LAMP assays performed with both LF and LB primers to accelerate the reaction can detect the target cells at concentrations on the order of 10^0 CFU per LAMP reaction tube (17, 20). On this basis, the sensitivity of our LAMP assays appears to be reasonable. As shown in Fig. 2c, the turbidity for 5.0×10^{-1} CFU per LAMP tube decreased to less than zero. A possible explanation for this phenomenon is the gradual sedimentation of the extracted APW enrichment broth cultures containing food components during LAMP because this phenomenon was also observed in our previous studies using DNA templates from fecal and food samples (17–19). In contrast, this phenomenon was never observed in our previous studies (17–19) or the present study when DNA templates from pure bacterial cultures were used.

When dilutions of AQ3815, AQ4037, AT4, and Vp02 cells in mixtures containing different amounts of the bacteria were used, the sensitivities of the two sets of multiplex LAMP assays (one detecting the *tdh*, *trh*1, and *trh*2 genes and the other detecting the *trh*1 and *trh*2 genes) were not decreased. The sensitivities of the individual *tdh*, *trh*1, and *trh*2 LAMP assays were affected, however, with 2-, 8-, and 2-fold decreases, respectively (Table 4), possibly due to genetic similarities of the three target sequences. On this basis, the multiplex LAMP assays would be useful for detecting virulent strains in seafood samples containing *tdh*, *trh*1, and *trh*2 at concentrations of 1.7, 170.4, and 10.0 CFU per LAMP reaction tube, corresponding to 9.2×10^1, 9.2×10^3, and 5.4×10^2 CFU per ml in APW enrichment cultures, respectively.

Risk assessment for *V. parahaemolyticus* in seafood is an increasingly important issue worldwide. However, current methods for quantitative detection of virulent strains are inconvenient and time-consuming (16). A method combining a three-tube most-probable-number culture method and PCR examination for virulent strains is useful but cumbersome (16). The sensitivity of the LAMP assay is at least 10-fold higher than that of a conventional PCR assay and typically equivalent to that of a real-time PCR assay (17–20). The high sensitivity of our LAMP assay and the fact that the amplification reaction results can be judged by simple visual assessment (Fig. 2) make it a more attractive tool for risk assessment, and future studies based on this characteristic would likely make a valuable contribution. Worldwide, outbreaks caused by virulent *V. parahaemolyticus* are frequent, highlighting the need for effective control of contaminants in seafood. The LAMP assay is a rapid, simple, and practical method which may facilitate surveillance for virulent *V. parahaemolyticus* contamination in seafood, screening of contaminated seafood samples before consumption, and investigations to determine the causative agents of food poisoning, as well as ecological studies associated with environmental factors, seasons, regions, and practices.

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