Development and Evaluation of a Rapid, Simple, and Sensitive Immunochromatographic Assay To Detect Thermostable Direct Hemolysin Produced by *Vibrio parahaemolyticus* in Enrichment Cultures of Stool Specimens

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Thermostable direct hemolysin (TDH) is considered to be a major virulence factor in *Vibrio parahaemolyticus*, and most cases of *V. parahaemolyticus* diarrhea in humans are caused by *tdh* gene-positive strains. In the present study, we developed an immunochromatographic assay to detect TDH (TDH-ICA) and evaluated the utility of TDH-ICA for the diagnosis of *V. parahaemolyticus* diarrhea. TDH-ICA allowed the detection of 0.2 ng/ml of TDH within 10 min. Fecal homogenates were spiked with various numbers of *tdh*-positive *V. parahaemolyticus* organisms, and their enrichment cultures were tested with TDH-ICA. The results of detection of TDH in the enrichment cultures by TDH-ICA were in accord with the results of recovery of the spiked *V. parahaemolyticus* organisms from the enrichment cultures by plating onto thiosulfate-citrate-bile salts-sucrose agar. When enrichment cultures of 217 stool specimens from patients with diarrhea were tested with TDH-ICA, the TDH-ICA results showed 100% sensitivity and specificity compared to the results of isolation of *V. parahaemolyticus* from the stool specimens by a conventional bacterial culture test. Since TDH-ICA was able to detect TDH in a fecal enrichment culture within 10 min, TDH-ICA testing of a fecal enrichment culture could be completed rapidly and easily within approximately 16 h, including incubation time for the fecal enrichment culture. These results indicate that TDH-ICA is a rapid, simple, and sensitive TDH detection method and that TDH-ICA testing of a fecal enrichment culture is useful as an adjunct to facilitate the early diagnosis of *V. parahaemolyticus* diarrhea.

Vibrio parahaemolyticus is a major food-borne pathogen that causes diarrhea in humans through seafood consumption, but not all strains are considered to be pathogenic. Thermostable direct hemolysin (TDH) has been found to be closely associated with the enteropathogenicity of *V. parahaemolyticus* (5). In the late 1980s, cases of diarrhea caused by *Vibrio parahaemolyticus* strains that did not produce TDH were found (8). The strains produced a new hemolysin that was immunologically similar but not identical to TDH. The hemolysin was referred to as TDH-related hemolysin (TRH). Currently, TDH and TRH are regarded as important virulence factors of *V. parahaemolyticus* (5, 13), and therefore, strains possessing the *tdh* gene encoding TDH and/or the *trh* gene encoding TRH are considered pathogenic strains (14). Since most cases (approximately 90%) of *V. parahaemolyticus* diarrhea in humans are caused by *tdh*-positive strains, including strains that possess both the *tdh* and *trh* genes (1, 2, 16, 17, 18), *tdh*-positive *V. parahaemolyticus* is a major causative organism of *V. parahaemolyticus* diarrhea.

Cases of *V. parahaemolyticus* diarrhea in humans are commonly diagnosed by isolating this pathogen from a stool specimen by a bacterial culture test. The test procedure includes the plating of stool or its enrichment culture onto selective

agar medium and the subsequent identification of suspected colonies on the agar medium. The identification includes biochemical tests, serotyping of O and K antigens, and examination for the presence of the *tdh* and *trh* genes (12, 20). Since the bacterial culture test requires at least 3 days and a complicated and time-consuming procedure, a simple and rapid method for the diagnosis of *V. parahaemolyticus* diarrhea has been desired.

Virulence factors are regarded as useful markers for the detection of pathogenic bacteria because they are almost completely specific for a particular pathogenic bacterium. For example, in the case of *V. parahaemolyticus* diarrhea, when a stool specimen containing *tdh*-positive *V. parahaemolyticus* is cultured in enrichment broth medium, the pathogen is expected to release TDH into the fecal enrichment culture, because TDH is a bacterial exotoxin. TDH is highly specific to *tdh*-positive *V. parahaemolyticus*, although some *Vibrio cholerae* non-O1 and *Vibrio mimicus* strains and all strains of *Vibrio hollisae* have been reported to produce hemolysins similar to TDH (22, 23, 25). Therefore, the occurrence of TDH in fecal enrichment culture is regarded as a useful marker for the detection of *tdh*-positive *V. parahaemolyticus* isolates in stool specimens. Since most cases of *V. parahaemolyticus* diarrhea are caused by *tdh*-positive strains (1, 2, 16, 17, 18), the detection of TDH in fecal enrichment cultures appears to be useful as an adjunct to the diagnosis of *V. parahaemolyticus* diarrhea. For this purpose, a rapid, simple, and sensitive method to detect TDH is necessary. Several methods, including the latex agglutination assay (KAP-RPLA; Denka Seiken Co., Ltd., Tokyo, Japan) and enzyme-

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linked immunosorbent assays (ELISAs) (6, 9, 10), have already been used for the detection of TDH. Each method has advantages and disadvantages. KAP-RPLA is commercially available and simple to perform, but it lacks sensitivity and requires a long incubation time before the test results are available. On the other hand, although ELISAs have excellent sensitivity, they require a complicated and time-consuming procedure. Considering the limitations mentioned above, the conventional TDH detection methods appear to be unsuitable for rapid, simple, and sensitive detection of TDH in fecal enrichment cultures.

The purpose of the present study was to develop a rapid, simple, and sensitive method to detect TDH and to evaluate the utility of the new method for the diagnosis of *V. parahaemolyticus* diarrhea. Using the combination of an immunochromatographic technique with monoclonal antibodies (MAbs) to TDH, a method that can detect levels of TDH in picograms per milliliter within 10 min was successfully developed. Here, we report in detail the development and the evaluation of an immunochromatographic assay to detect TDH (TDH-ICA).

MATERIALS AND METHODS

Bacterial strains. A total of 133 *V. parahaemolyticus* strains used in this study, including 47 different serotypes of O and K antigens, were isolated from patients with diarrhea derived from 55 different food poisoning incidents that occurred in Osaka prefecture, Japan, between 1983 and 1995. These included 48 Kanagawa phenomenon (KP)-positive strains that possessed only the *tdh* gene, 46 KP-positive strains that possessed both the *tdh* and *trh* genes, 19 KP-negative strains that possessed both the *tdh* and *trh* genes, 10 KP-negative strains that possessed only the *trh* gene, and 10 KP-negative strains that did not possess either the *tdh* or the *trh* gene. The KP is a beta-type hemolysis that occurs on a special blood agar medium, Wagatsuma agar, and is induced by high-level production of TDH (14). These strains were maintained in preservation medium (1% Bacto peptone [Becton Dickinson Microbiology Systems, Sparks, MD], 0.4% Bacto agar [Becton Dickinson], 2% NaCl, pH 7.4) at room temperature until use. Strains of *Citrobacter freundii*, *Escherichia coli*, *Plesiomonas shigelloides*, *Salmonella enteritidis*, *Shigella sonnei*, *V. cholerae* O1, *V. mimicus*, *V. hollliae*, and *Vibrio vulnificus* were also obtained from clinical samples. *V. cholerae* non-O1 strains were isolated at the Kansai Airport Quarantine Station, Osaka, Japan, from patients with traveler's diarrhea. *Aeromonas hydrophila* (IAM1646), *Aeromonas sobria* (IAM12333), *Enterobacter cloacae* (IAM12349T), and *Pseudomonas aeruginosa* (IAM1514T) were provided by the Institute of Applied Microbiology culture collection (Tokyo, Japan). *V. alginolyticus* (IFO15630T) was obtained from the Institute for Fermentation (Osaka, Japan).

Production of MAbs to TDH. TDH was purified from Kanagawa hemolysin (Sigma Chemical Co., St. Louis, MO) using a modification of a protocol described previously by Huntley et al. (11). Briefly, Kanagawa hemolysin was subjected to ion-exchange chromatography with a monoQ column (Amersham Biosciences, Piscataway, NJ) and was fractionated with an NaCl gradient of 0 to 1 M. The peak fractions (detected by measurement of the A_{280}) were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, pooled, concentrated, and loaded onto a Superose12 gel filtration column (Amersham). The purified TDH was recognized on the basis of its molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was used as an immunogen for the production of MAbs and as an antigen in ELISAs. Three female BALB/c mice (8 weeks old) were immunized intraperitoneally with 20 μ g of the purified TDH emulsified in Freund's complete adjuvant (Wako Pure Chemical Industries, Osaka, Japan). After 2, 4, and 6 weeks, the mice were boosted intraperitoneally with the same antigen emulsified in Freund's incomplete adjuvant (Wako). At 10 weeks, a final immunization was carried out intraperitoneally with the same antigen alone. Three days after the final immunization, splenocytes of the mice and P3-X63-Ag8.U1 myeloma cells were mixed at a ratio of 5:1 and fused with 40% polyethylene glycol by a modification of a protocol described previously by Galfre and Milstein (3). The resulting hybridoma cells were selected in a hypoxanthine-aminopterin-thymidine medium, and the culture supernatants of actively growing hybridoma cells were then screened for the production of antibody by an ELISA using the purified TDH as the coating antigen. The positive hybridoma cells identified by ELISA were cloned twice by limiting dilution and then amplified in the ascites of pristine (2,6,10,14-tetramethylpen-

tadecane)-primed mice. MAbs were purified from the ascites supernatant by affinity chromatography on a protein A-agarose gel (Affi-Gel Protein A MAPS II kit; Bio-Rad Laboratories, Hercules, CA) after salting out with 50% saturated ammonium sulfate (pH 7.4). Isotyping of MAbs was performed with a mouse monoclonal antibody isotyping kit (IsoStrip; Roche Diagnostics, Indianapolis, IN).

Determination of the best combination of MAbs for TDH-ICA by sandwich ELISA. All of the obtained MAbs were biotinylated with EZ-Link NHS-LC-LC-Biotin (PIERCE, Rockford, IL) for use as the labeling antibody of the sandwich ELISA. Each well of a 96-well ELISA plate (Sumitomo Bakelite Co., Tokyo, Japan) was coated with 100 μ l of an unlabeled MAb solution (100 μ g/ml in 50 mM sodium bicarbonate buffer, pH 9.6) overnight at 4°C, and the unbound active sites in each well were then blocked with phosphate-buffered saline (PBS) containing 20% horse serum for 1 h at room temperature. After three washes of each well with PBS containing 0.05% Tween 20 (PBS-T), 100 μ l of a purified TDH solution (10 ng/ml in PBS-T) and 50 μ l of a biotinylated MAb solution (10 μ g/ml in PBS-T) were added to each well and incubated for 1 h at room temperature. After four washes with PBS-T, 100 μ l of a peroxidase-avidin complex solution prepared according to the manufacturer's instruction manual (VECTASTAIN Elite ABC kit; Vector, Burlingame, CA) was added and incubated for 1 h at room temperature. After five washes with PBS-T, the peroxidase activity in each well was detected by the addition of 3,3',5,5'-tetramethylbenzidine solution (1 mg of 3,3',5,5'-tetramethylbenzidine and 1.5 μ l of 30% H_2O_2 in 10 ml of 50 mM citrate buffer, pH 5.5) as a substrate, followed by incubation for 10 min at room temperature. After the reaction was stopped by the addition of 1 M H_2SO_4 solution, the absorbance of each well at 450 nm was measured using an EIA microplate reader (model 550; Bio-Rad). Finally, the absorbance values of wells were compared with each other, and the combination of MAbs used in the well that yielded the highest absorbance value was used for TDH-ICA.

TDH-ICA. All solutions and buffers used to prepare the test strip and the colloidal gold conjugate were filtered with 0.22- μ m-pore-size membrane filters before use.

The test strips were prepared by using a HiFlowPlus Development Kit-15 (Millipore, Bedford, MA). Briefly, an absorbent pad was attached to a laminated membrane card (HiFlow Plus 135) so that the pad slightly overlapped the membrane, and the assembly was then cut into strips consisting of the membrane (5 by 25 mm) with the absorbent pad (5 by 17 mm). A 1.0- μ l aliquot of MAB solution (2 mg/ml in 5 mM borate buffer, pH 8.5) was deposited on the membrane as a 1-mm-wide line at about the midpoint of the length of the membrane as the detection zone of TDH by using a 10- μ l syringe. As the control zone, 1.0 μ l of goat anti-mouse immunoglobulin G solution (2 mg/ml in 5 mM borate buffer, pH 8.5) (Sigma) was deposited on the membrane as a 1-mm-wide line 5 mm downstream from the detection zone. After drying for 1 h at 37°C and then drying overnight at 4°C, the test strips were stored at 4°C until use.

To prepare the colloidal gold conjugate, 0.3 ml of a MAB solution (0.1 mg/ml in 2 mM borax buffer, pH 9.0) was added to 10 ml of a colloidal gold suspension (40 nm; BBI International, Cardiff, United Kingdom) adjusted to pH 9.0 by the addition of 100 mM K_2CO_3 solution. After stirring for 30 min at room temperature, 1.0 ml of a 10% bovine serum albumin (BSA) solution (in 2 mM borax buffer, pH 9.0) was added to the mixture. After stirring for 30 min at room temperature, the mixture was centrifuged at $4,500 \times g$ at 5°C for 10 min. The resulting pellet was suspended in 2 mM borax buffer (pH 9.0) containing 1% BSA, and the suspension was then centrifuged at $4,500 \times g$ at 5°C for 10 min. The final pellet (the MAB-gold conjugate) was resuspended in 50 mM Tris-HCl-150 mM NaCl buffer (pH 8.2) containing 1% BSA and 20% glycerol and then stored at -25°C until use.

TDH-ICA testing of samples was performed according to the following procedure. Twenty-five microliters of a sample and 25 μ l of the MAB-gold conjugate (diluted 1:10 in 100 mM Tris-HCl buffer, pH 8.0, containing 0.5% BSA and 2% Triton X-100) were added to a well of a 96-well general assay plate (flat-bottom type) (Corning, Acton, MA). After immediate mixing by repeated aspiration and ejection with a micropipette, the test strip was inserted into the mixture in the well. After migration of the mixture through the membrane for 10 min at room temperature, the appearance of red lines at both the detection zone and the control zone was interpreted as positive detection of TDH (Fig. 1).

TDH-ICA testing of broth cultures of *V. parahaemolyticus* and other bacterial strains. The test strains were inoculated into 2 ml of alkaline peptone water containing 2% NaCl (APW) (Eiken kizai Co., Ltd., Tokyo, Japan). All cultures were incubated for 16 h at 37°C without shaking and then centrifuged at $10,000 \times g$ at 5°C for 5 min. The resulting supernatants were tested with TDH-ICA and KAP-RPLA (Denka Seiken).

FIG. 1. Detection of TDH by TDH-ICA. A positive result is indicated by the appearance of red lines at both the detection zone and the control zone of the test strip.

TDH-ICA testing of enrichment cultures of *V. parahaemolyticus*-spiked fecal homogenates. A *V. parahaemolyticus* strain (serotype O1:K69), which possesses both the *tdh* and *trh* genes and yielded a KP-negative result on Wagatsuma agar (13), was used for the preparation of spiked fecal homogenates. This strain was estimated to produce TDH at a very low level because it is *tdh* positive but KP negative (14). The *tdh*-positive but KP-negative strain was incubated in 2 ml of APW at 37°C for 16 h. The resulting culture was estimated to contain approximately 2×10^8 CFU/ml by colony counting on marine agar plates (Becton Dickinson). Serial 10-fold dilutions of the culture were made in PBS and were spiked into 10% fecal homogenate (in PBS) to yield concentrations of 2×10^1 to 10^5 CFU per 1 ml of the fecal homogenate (equivalent to 0.1 g of stool). A loopful of each of the spiked fecal homogenates and the fecal homogenate as 0 CFU/ml was inoculated into 20 ml of APW, which was incubated at 37°C for 16 h without shaking. After centrifugation at $10,000 \times g$ at 5°C for 5 min, the resulting supernatants were tested with TDH-ICA. In parallel, a loopful of each of the spiked fecal homogenates, the fecal homogenate, and their 16-h APW cultures was streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar medium (Eiken Kizai). After incubation at 37°C for 16 h, the resulting sucrose-nonfermenting colonies (green colonies) were identified as *V. parahaemolyticus*.

TDH-ICA testing of enrichment cultures of stool specimens from patients with diarrhea. A total of 217 stool specimens were obtained from 217 patients with diarrhea derived from 24 different food poisoning incidents that occurred in Osaka prefecture, Japan, between July and October 2004. The specimens, all of which were diarrheal stool samples, were transported to our laboratory from 13 local public health centers in Osaka prefecture and were subjected to bacteriological examination for enteropathogens, including *V. parahaemolyticus*, within 2 h from the time they arrived in our laboratory. For TDH-ICA testing of fecal enrichment cultures, a loopful of each of the stool specimens was inoculated into 20 ml of APW, which was incubated at 37°C for 16 h without shaking. After centrifugation at $10,000 \times g$ at 5°C for 5 min, the resulting supernatants were tested using TDH-ICA. In parallel, for the isolation of *V. parahaemolyticus*, a loopful of each of the stool specimens was streaked onto TCBS agar, which was then incubated at 37°C for 16 h. If the 16-h TCBS agar culture was negative for sucrose-nonfermenting colonies (green colonies), a loopful of the 16-h APW culture of the stool specimen was streaked onto TCBS agar. The sucrose-nonfermenting colonies on TCBS agar were screened for *V. parahaemolyticus* by a battery of biochemical tests, with positive results in the tests for fermentation without gas production of glucose, for lysine decarboxylase, for the production of indole from tryptophan, and for growth in 3, 6, and 8% NaCl and with negative results for fermentation of sucrose and lactose and for growth in 0 and 10% NaCl. The presumptively identified strains were subjected to the serotyping of O and K antigens using a commercially available rabbit antiserum (Denka Seiken) to determine whether they were *V. parahaemolyticus* strains. All of the isolated *V. parahaemolyticus* strains were examined for the presence of the *tdh* and *trh* genes by the PCR method (19).

RESULTS

Production of MAbs to TDH. A total of 480 wells contained hybridoma cells obtained by the fusion of splenocytes from three immunized mice and P3U1 myeloma cells. Of these wells, culture supernatants of 14 wells yielded positive results by ELISA. By cloning of the hybridoma cells in the positive wells by limiting dilution, five stable hybridoma cell lines secreting MAbs to TDH (4D3, 4E5, 4C4, 3D2, and 2G9) were finally obtained. All MAbs from the cell lines belonged to the immunoglobulin G1(κ) subclass. To select a combination of MAbs for use in a sensitive immunochromatographic test to detect TDH, we screened coating and labeling antibody combinations of the five MAbs for detection sensitivity for TDH in the sandwich ELISA. The results revealed that the sandwich ELISA using MAb 3D2 as the coating antibody and MAb 4E5 as the labeling antibody yielded the highest absorbance value when 1 ng of purified TDH was assayed. Since the range of absorbance values in the sandwich ELISA reflects the detection sensitivity for TDH, the sandwich ELISA that yielded the highest absorbance value has the highest sensitivity for the detection of TDH. The above-described results, therefore, mean that the sandwich ELISA using the combination of MAbs 3D2 and 4E5 had the highest sensitivity for the detection of TDH. MAbs 3D2 and 4E5 were therefore considered to be the best combination for a sensitive immunochromatographic test to detect TDH.

Establishment of TDH-ICA. By using MAb 3D2 as the antibody immobilized on test strips and MAb 4E5 as the antibody conjugated with colloidal gold particles, a sandwich-type immunochromatographic assay to detect TDH (TDH-ICA) was established. To determine the minimum detectable concentration of TDH, each TDH solution at various concentrations (0.1, 0.2, 0.5, 1, 100, and 10,000 ng of TDH/ml) and PBS as the zero concentration were tested with TDH-ICA. The red line produced by capturing the MAb-gold conjugate was observed at the control zone of all test strips within 10 min, suggesting that the MAb-gold conjugate migrated by capillary action through the membrane beyond the detection zone without aggregation. Another red line appeared at the detection zone of the test strip within 10 min due to the production of the sandwich immunocomplex of TDH and two MAbs when the concentration of TDH was 0.2 ng/ml or higher. In addition, the detectable concentration range of TDH-ICA extended to 10,000 ng/ml without false-negative results due to the prozone phenomenon. These results indicate that TDH-ICA has a minimum detectable concentration of 0.2 ng of TDH/ml and a wide dynamic range. The test strips of TDH-ICA could be used for at least 4 months when stored at 4°C.

TDH-ICA testing of broth cultures of *V. parahaemolyticus* and other bacterial strains. Although the *tdh* gene of *V. parahaemolyticus* has some variants, their nucleotide sequences are well conserved (>97% identity), and their protein products are immunologically indistinguishable (14). However, we considered it necessary to examine whether TDH-ICA could detect TDH produced by various *tdh*-positive strains of *V. parahaemolyticus* before using TDH-ICA as a TDH detection method. For this purpose, broth cultures of 113 *tdh*-positive strains of *V. parahaemolyticus* were tested with TDH-ICA. Broth cultures of 10 *tdh*-negative, *trh*-negative strains of *V. parahaemolyticus*

TABLE 1. Comparison between TDH-ICA results and KAP-RPLA results obtained for broth cultures of *Vibrio parahaemolyticus* and other bacterial strains

Test bacterium	Presence of <i>tdh</i> / <i>trh</i> ^a	KP ^b	No. of strains tested	No. (%) of strains positive by:	
				TDH-ICA ^c	KAP-RPLA ^d
<i>Vibrio parahaemolyticus</i>	+/-	+	48	48 (100)	48 (100)
	+/+	+	46	46 (100)	46 (100)
	+/+	-	19	19 (100)	0 (0)
	-/+	-	10	0 (0)	0 (0)
	-/-	-	10	0 (0)	0 (0)
<i>Vibrio cholerae</i> non-O1	-/-	NT	10	0 (0)	0 (0)
<i>Vibrio hollisae</i>	+/-	NT	11	0 (0)	0 (0)
<i>Vibrio mimicus</i>	+/-	NT	2	2 (100)	2 (100)
	-/-	NT	10	0 (0)	0 (0)
<i>Aeromonas hydrophila</i>	NT	NT	1	0 (0)	0 (0)
<i>Aeromonas sobria</i>	NT	NT	1	0 (0)	0 (0)
<i>Citrobacter freundii</i>	NT	NT	1	0 (0)	0 (0)
<i>Enterobacter cloacae</i>	NT	NT	1	0 (0)	0 (0)
<i>Escherichia coli</i>	NT	NT	1	0 (0)	0 (0)
<i>Plesiomonas shigelloides</i>	NT	NT	1	0 (0)	0 (0)
<i>Pseudomonas aeruginosa</i>	NT	NT	1	0 (0)	0 (0)
<i>Salmonella enteritidis</i>	NT	NT	1	0 (0)	0 (0)
<i>Shigella sonnei</i>	NT	NT	1	0 (0)	0 (0)
<i>Vibrio alginolyticus</i>	NT	NT	1	0 (0)	0 (0)
<i>Vibrio cholerae</i> O1	NT	NT	1	0 (0)	0 (0)
<i>Vibrio vulnificus</i>	NT	NT	1	0 (0)	0 (0)

^a +, presence; -, absence; NT, not tested.^b KP, Kanagawa phenomenon; +, positive; -, negative; NT, not tested.^c TDH-ICA, immunochromatographic assay to detect TDH.^d KAP-RPLA, latex agglutination assay to detect TDH (Denka Seiken).

were tested as negative controls. The results are summarized in Table 1. The 113 *tdh*-positive strains included 39 different serotypes of O and K antigens and were derived from 55 different food poisoning incidents. The amount of TDH production by *tdh*-positive *V. parahaemolyticus* strains differs from strain to strain, and *tdh*-positive but KP-negative strains produce TDH at very low levels (14). The 113 *tdh*-positive strains, therefore, included 19 *tdh*-positive but KP-negative strains for the purpose of evaluating the ability of TDH-ICA to detect the small amount of TDH produced by *tdh*-positive but KP-negative strains. When broth cultures of the 113 *tdh*-positive strains were tested with KAP-RPLA, TDH was detected in broth cultures of the 94 *tdh*-positive, KP-positive strains but not in broth cultures of the 19 *tdh*-positive but KP-negative strains. This result revealed that the amounts of TDH produced by the 19 *tdh*-positive but KP-negative strains were very small. TDH-ICA, however, was able to detect TDH in all broth cultures of the 113 *tdh*-positive strains regardless of their KP reaction. TDH-ICA yielded negative results for all broth cultures of the *tdh*-negative, *trh*-negative strains tested as negative controls.

To examine whether TDH-ICA can detect TRH of *V. parahaemolyticus*, broth cultures of 10 strains of *V. parahaemolyticus* that were *trh* positive only were tested with TDH-ICA and KAP-RPLA. As shown in Table 1, all of the broth cultures yielded negative results with both TDH-ICA and KAP-RPLA. Since KAP-RPLA has a minimum detectable concentration of 10 ng/ml of TRH (21), this result indicates that the TRH concentrations that were produced by the strains in the broth cultures were less than 10 ng/ml and that TDH-ICA could not detect them.

TABLE 2. Comparison between TDH-ICA results and culture results obtained for fecal homogenates spiked with *tdh*-positive but KP-negative *Vibrio parahaemolyticus*

No. of spiked organisms (CFU/ml of fecal homogenate)	TDH-ICA result ^a	Result of culture on TCBS agar ^b	
		Direct plating	Plating of enrichment culture
2.0×10^5	+	+	+
2.0×10^4	+	+	+
2.0×10^3	+	+	+
2.0×10^2	+	-	+
2.0×10^1	-	-	-
0	-	-	-

^a All tests were performed for enrichment cultures of fecal homogenates with APW. +, TDH-positive detection; -, TDH-negative detection.^b +, positive (green colonies of *V. parahaemolyticus*); -, negative.

In addition to *tdh*-positive *V. parahaemolyticus* strains, some *V. cholerae* non-O1 and *V. mimicus* strains and all strains of *V. hollisae* have been reported to possess the *tdh* gene (14) and to produce hemolysin similar to TDH (NAG-rTDH, Vm-rTDH, and Vh-rTDH are used throughout the text to denote hemolysin similar to TDH produced by *V. cholerae* non-O1 strains, *V. mimicus* strains, and *V. hollisae* strains, respectively) (22, 23, 25). Of these bacterial strains, broth cultures of 11 *tdh*-positive strains of *V. hollisae* and 2 *tdh*-positive strains of *V. mimicus* were tested with TDH-ICA and KAP-RPLA. The results are summarized in Table 1. The *tdh*-positive strains of *V. mimicus* produced Vm-rTDH detectable by TDH-ICA and KAP-RPLA in the broth cultures. This result indicates that TDH-ICA has cross-reactivity with Vm-rTDH from *V. mimicus*. In contrast, the *tdh*-positive strains of *V. hollisae* did not grow well and did not produce Vh-rTDH that was detectable by TDH-ICA and KAP-RPLA in the broth cultures when they were cultured with APW. In the preliminary study, 154 clinical *V. cholerae* non-O1 strains that were isolated at the Kansai Airport Quarantine Station, Osaka, Japan, from 154 patients with traveler's diarrhea were examined with the PCR method (19), which can also detect also the *tdh* gene of *V. cholerae* non-O1 strains. The results, however, indicated that all of the strains were negative for the presence of the *tdh* gene (data not shown). Since *tdh*-positive *V. cholerae* non-O1 strains could not be found, their broth cultures were not tested with TDH-ICA. As shown in Table 1, when broth cultures of the *tdh*-negative *V. cholerae* non-O1 and *V. mimicus* strains were tested with TDH-ICA, they all yielded negative results.

TDH-ICA testing of enrichment cultures of *V. parahaemolyticus*-spiked fecal homogenates. To evaluate the ability of TDH-ICA to detect TDH in enrichment cultures of stool specimens containing *tdh*-positive *V. parahaemolyticus* strains, enrichment cultures of the fecal homogenates spiked with various numbers of *tdh*-positive *V. parahaemolyticus* organisms were tested with TDH-ICA. The TDH-ICA results were compared with the culture results of the spiked fecal homogenates and their enrichment cultures on TCBS agar. As shown in Table 2, the results of detection of TDH in the enrichment cultures by TDH-ICA were in accord with the results of recovery of the spiked *V. parahaemolyticus* organisms from the enrichment cultures by plating onto TCBS agar. TDH-ICA was able to detect TDH in enrichment cultures of the spiked fecal homogenates.

TABLE 3. Comparison between TDH-ICA results and results of isolation of *V. parahaemolyticus* obtained for 217 stool specimens from patients with diarrhea as a result of food poisoning incidents

No. of specimens	Isolation of <i>V. parahaemolyticus</i> ^a		No. of specimens with the indicated result by TDH-ICA ^b	
	Direct plating of stool	Plating of fecal enrichment culture	Positive	Negative
39 ^c	+	NT	39	0
11 ^c	—	+	11	0
167 ^d	—	—	0	167

^a +, positive isolation; —, negative isolation; NT, not tested.

^b All tests were performed for fecal enrichment cultures with APW.

^c All of the isolated *V. parahaemolyticus* strains possessed only the *tdh* gene.

^d The following enteropathogens were isolated from the indicated number of specimens: *Campylobacter jejuni* (26 specimens), *Salmonella* spp. (22 specimens), *Bacillus cereus* (6 specimens), and *Staphylococcus aureus* (6 specimens).

enate even when the homogenate did not yield *V. parahaemolyticus* by direct plating onto TCBS agar. These results suggest that TDH-ICA testing of fecal enrichment cultures is sensitive enough to detect stool specimens containing *tdh*-positive *V. parahaemolyticus* strains, compared to a conventional bacterial culture.

TDH-ICA testing of enrichment cultures of stool specimens from patients with diarrhea. To evaluate the utility of TDH-ICA testing of fecal enrichment cultures for the diagnosis of *V. parahaemolyticus* diarrhea, enrichment cultures of 217 stool specimens obtained from patients with diarrhea as a result of food poisoning incidents were tested with TDH-ICA. The TDH-ICA results were compared with the results of isolation of *V. parahaemolyticus* from the stool specimens by a bacterial culture test. The results are summarized in Table 3. Of 217 stool specimens, *V. parahaemolyticus* was isolated from 50 stool specimens, which were derived from 11 different food poisoning incidents, by the bacterial culture test. The 50 stool specimens included 39 specimens that yielded *V. parahaemolyticus* organisms by direct plating of stools onto TCBS agar and 11 specimens that yielded this pathogen by plating of their enrichment cultures onto TCBS agar. All of the isolated *V. parahaemolyticus* strains possessed only the *tdh* gene. The bacterial culture test for the isolation and identification of *V. parahaemolyticus* from the stool specimens required 3 days when the stools yielded this pathogen by direct plating onto TCBS agar and one additional day when the stools yielded this pathogen by plating of their enrichment cultures onto TCBS agar. Among the 167 *V. parahaemolyticus*-negative specimens, *Campylobacter jejuni* was isolated from 26 specimens, *Salmonella* spp. were isolated from 22 specimens, *Bacillus cereus* was isolated from 6 specimens, and *Staphylococcus aureus* was isolated from 6 specimens. All enrichment cultures of the 50 stool specimens from which *V. parahaemolyticus* was isolated by the bacterial culture test yielded positive results with TDH-ICA. None of the enrichment cultures of the remaining 167 stool specimens that were negative for *V. parahaemolyticus* by the bacterial culture test yielded a positive result with TDH-ICA. Therefore, the TDH-ICA results were completely in agreement with the results of isolation of *V. parahaemolyticus* from the stool specimens by the conventional bacterial culture test and

showed 100% sensitivity (50 of 50 samples) and 100% specificity (167 of 167 samples). TDH-ICA testing of the fecal enrichment cultures could be completed within approximately 16 h, including the incubation time for the fecal enrichment culture with APW.

DISCUSSION

Most cases (approximately 90%) of *V. parahaemolyticus* diarrhea in humans are caused by *tdh*-positive strains (1, 2, 16, 17, 18). When a stool specimen containing *tdh*-positive *V. parahaemolyticus* is cultured in enrichment broth medium, the pathogen is expected to release TDH into the fecal enrichment culture. The presence of TDH in the fecal enrichment culture is regarded as a useful marker for the detection of stool specimens containing *tdh*-positive *V. parahaemolyticus* strains. For the detection of TDH in fecal enrichment cultures, we developed TDH-ICA, an immunochromatographic assay to detect TDH. TDH-ICA has a minimum detectable concentration of 0.2 ng/ml of TDH, which is lower than those of KAP-RPLA (Denka Seiken) and ELISAs (6, 9, 10). Since TDH-ICA is able to detect TDH in a fecal enrichment culture within 10 min, TDH-ICA testing of the fecal enrichment culture can be completed rapidly and simply within approximately 16 h, including the incubation time for the fecal enrichment culture. TDH-ICA is therefore considered to be more useful than the conventional TDH detection methods for the rapid, simple, and sensitive detection of TDH in fecal enrichment cultures. Since TDH-ICA testing of a fecal enrichment culture is a more rapid means for the diagnosis of *V. parahaemolyticus* diarrhea than a conventional bacterial culture test, the test results appear to be more useful for making a timely decision about the course of treatment of patients.

Before the application of TDH-ICA for the diagnosis of human diarrhea, it is very important to examine the cross-reactivity of TDH-ICA with other related hemolysins. In addition to *tdh*-positive *V. parahaemolyticus* strains, some non-*V. cholerae* O1 and *V. mimicus* strains and all strains of *V. hollisae* have been reported to possess the *tdh* gene (14) and to produce NAG-rTDH, Vm-rTDH, and Vh-rTDH, respectively, which are hemolysins similar to TDH (22, 23, 25). Of these hemolysins, TDH-ICA has cross-reactivity with Vm-rTDH from *V. mimicus* (Table 1). In the preliminary study, although 154 clinical *V. cholerae* non-O1 strains were examined by the PCR method (19), *tdh*-positive strains could not be found (data not shown). The incidence of the *tdh* gene among clinical *V. cholerae* non-O1 strains is very low (7, 15). Therefore, we could not test broth cultures of *tdh*-positive *V. cholerae* non-O1 strains with TDH-ICA. Since the amino acid compositions of TDH and NAG-rTDH from *V. cholerae* non-O1 strains are very similar (24), TDH-ICA is also expected to have cross-reactivity with NAG-rTDH. This cross-reactivity of TDH-ICA with NAG-rTDH or Vm-rTDH suggests that TDH-ICA would also yield positive results for enrichment cultures of stool specimens containing *tdh*-positive *V. cholerae* non-O1 or *tdh*-positive *V. mimicus* strains in addition to *tdh*-positive *V. parahaemolyticus* strains. *V. cholerae* non-O1 and *V. mimicus* strains, however, can be distinguished from *V. parahaemolyticus* strains based on the characteristics of their colonies on a chromogenic agar medium (4). Therefore, if TDH-ICA testing of a fecal

enrichment culture yields a positive result, whether the causative bacterium is *V. parahaemolyticus* can be determined easily based on the culture results of the same stool sample on a chromogenic agar medium. While most clinical strains of *V. parahaemolyticus* possess the *tdh* gene (1, 2, 16, 17, 18), the incidence of the *tdh* gene among clinical *V. cholerae* non-O1 and *V. mimicus* strains is very low (7, 15). Positive results with TDH-ICA testing of fecal enrichment cultures are therefore expected to almost always be due to *tdh*-positive *V. parahaemolyticus*. On the other hand, since the *tdh*-positive *V. hollisae* strains did not produce Vh-rTDH that was detectable by TDH-ICA in the broth cultures when they were cultured with APW (Table 1), TDH-ICA appears not to yield positive results for enrichment cultures with APW of stool specimens containing this pathogen.

As shown in Table 1, TDH-ICA could not detect TRH in broth cultures of the strains of *V. parahaemolyticus* that possessed only the *trh* gene when the strains were cultured with APW. The lack of reactivity of TDH-ICA with TRH or the lack of TRH production by the strains should be considered as a possible reason for this result. However, regardless of the reason, the result suggests that TDH-ICA would yield negative results for enrichment cultures with APW of stool specimens containing strains of *V. parahaemolyticus* that possessed only the *trh* gene. Therefore, TDH-ICA testing of a fecal enrichment culture is unsuitable as a replacement for a conventional bacterial culture to diagnose cases of human diarrhea caused by strains of *V. parahaemolyticus* that possess only the *trh* gene. However, since most cases of *V. parahaemolyticus* diarrhea are caused by *tdh*-positive strains (1, 2, 16, 17, 18), TDH-ICA testing of a fecal enrichment culture appears to be useful as an adjunct to the diagnosis of *V. parahaemolyticus* diarrhea.

Among the *tdh*-positive strains of *V. parahaemolyticus*, the quantity of TDH produced differs greatly from strain to strain, and *tdh*-positive but KP-negative strains of *V. parahaemolyticus* produce TDH at very low levels (14). Therefore, when a stool specimen that contains *tdh*-positive but KP-negative *V. parahaemolyticus* is cultured in enrichment broth medium, the amount of TDH that is produced by the pathogen in the fecal enrichment culture is expected to be very small. For the diagnosis of *V. parahaemolyticus* diarrhea using the presence of TDH in a fecal enrichment culture as a marker, TDH-ICA is needed to detect the very small amount of TDH produced by *tdh*-positive but KP-negative *V. parahaemolyticus* organisms in the fecal enrichment culture. We therefore evaluated TDH-ICA testing using enrichment cultures of fecal homogenates spiked with various numbers of *tdh*-positive but KP-negative *V. parahaemolyticus* organisms (Table 2). The results suggested that TDH-ICA testing of a fecal enrichment culture is sensitive enough to detect stool specimens containing *tdh*-positive but KP-negative *V. parahaemolyticus* strains, compared to a conventional bacterial culture. Therefore, TDH-ICA testing of a fecal enrichment culture also appears to be suitable for diagnosing cases of human diarrhea caused by *tdh*-positive but KP-negative *V. parahaemolyticus* strains.

The direct detection of TDH in stool specimens would be a more rapid and simple means for the diagnosis of *V. parahaemolyticus* diarrhea than the detection of TDH in fecal enrichment cultures. The direct detection of TDH in stool specimens using the ELISA system was evaluated for its utility in the

diagnosis of *V. parahaemolyticus* diarrhea in a previous study (6). The results, however, revealed that the results of direct detection of TDH in stool specimens were not completely compatible with the results of isolation of *tdh*-positive *V. parahaemolyticus* from the stool specimens. In the present study, when 217 stool specimens from patients with diarrhea were examined with a bacterial culture test, *tdh*-positive *V. parahaemolyticus* was isolated from 50 stool specimens (Table 3). Although we also tested all of the 50 stool specimens directly by TDH-ICA, only 16 stool specimens yielded positive results (data not shown). These results reveal that the direct detection of TDH in stool specimens is an unreliable means for the diagnosis of *V. parahaemolyticus* diarrhea. Clarification of the reason for discrepancies between the results of the direct detection of TDH in stool specimens and those of bacterial culture will require additional experiments. In contrast, when enrichment cultures of the 217 stool specimens were tested with TDH-ICA, the test results showed 100% sensitivity (50 of 50 samples) and 100% specificity (167 of 167 samples) compared with the results of isolation of *V. parahaemolyticus* from the stool specimens by a conventional bacterial culture test (Table 3). This result indicates that TDH-ICA testing of a fecal enrichment culture is a more reliable means for the diagnosis of *V. parahaemolyticus* diarrhea than direct TDH-ICA testing of a stool specimen. Although TDH-ICA testing of a fecal enrichment culture is less rapid and simple than direct TDH-ICA testing of a stool specimen, the total time (approximately 16 h) for the assay is considerably shorter than that (at least 3 days) required for a conventional bacterial culture test. In addition, TDH-ICA testing of a fecal enrichment culture is a simpler procedure than a conventional bacterial culture test.

In conclusion, the presence of TDH in fecal enrichment cultures is regarded as a useful marker for the detection of stool specimens containing *tdh*-positive *V. parahaemolyticus* strains. TDH-ICA is a rapid, simple, and sensitive TDH detection method and is suitable for the detection of this marker. TDH-ICA testing of a fecal enrichment culture is useful as an adjunct to facilitate the early diagnosis of *V. parahaemolyticus* diarrhea.

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