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Soft-Agar-Coated Filter Method for Early Detection of Viable and Thermostable Direct Hemolysin (TDH)- or TDH-Related Hemolysin-Producing *Vibrio parahaemolyticus* in Seafood

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A novel method for detecting viable and thermostable direct hemolysin (TDH)-producing or TDH-related hemolysin (TRH)-producing *Vibrio parahaemolyticus* in seafood was developed. The method involved (i) enrichment culture, selective for viable, motile cells penetrating a soft-agar-coated filter paper, and (ii) a multiplex PCR assay targeting both the TDH gene (*tdh*) and TRH gene (*trh*) following DNase pretreatment on the test culture to eradicate any incidental DNAs that might have been released from dead cells of *tdh*- or *trh*-positive (*tdh*$^+$ *trh*$^+$) strains and penetrated the agar-coated filter. A set of preliminary laboratory tests performed on 190 ml of enrichment culture that had been inoculated simultaneously with ca. 100 viable cells of a strain of *tdh*$^+$ *trh*$^+$ *V. parahaemolyticus* and dense populations of a viable strain of *tdh*- and *trh*-negative *V. parahaemolyticus* or *Vibrio alginolyticus* indicated that the method detected the presence of viable *tdh*$^+$ *trh*$^+$ strains. Another set of preliminary tests on 190 ml of enrichment culture that had been initially inoculated with a large number of dead cells of the *tdh*$^+$ *trh*$^+$ strain together with dense populations of the *tdh*- and *trh*-negative strains confirmed that the method did not yield any false-positive results. Subsequent quasi-field tests using various seafood samples (ca. 20 g), each of which was experimentally contaminated with either or both hemolysin-producing strains at an initial density of ca. 5 to 10 viable cells per gram, demonstrated that contamination could be detected within 2 working days.

Vibrio parahaemolyticus is a motile gram-negative rod-shaped, halophilic, facultative anaerobe that naturally inhabits estuaries and their fauna worldwide. The bacterium can cause one of the major food-borne gastroenteric infections, often associated with the consumption of raw or under-cooked seafood (2). Past epidemiological studies (6, 7, 16) revealed a strong association between gastroenteritis and both thermostable direct hemolysin (TDH) and another hemolysin, termed TDH-related hemolysin (TRH), produced by *V. parahaemolyticus*. Both hemolysins are thus considered to be major virulence factors of *V. parahaemolyticus*. Structural genes for TDH and TRH, *tdh* and *trh*, are encoded chromosomally, and PCR-based methods to detect the genes have been successfully developed (17, 19).

TDH- or TRH-producing *V. parahaemolyticus* is usually found together with much larger populations of avirulent strains in the environment, and it has therefore been technically difficult to detect these virulent strains in seafood by conventional culture methods (18). In this context, the Food and Drug Administration (4) recommended a limit of 100 cells of *V. parahaemolyticus* per gram of seafood, expressed as the most probable number (MPN), based at least in part on the assumption that seafood below such a contamination level might not contain the virulent strain. However, the recommended MPN technique requires 4 to 7 days to complete, thereby posing a practical problem for the food safety program to be performed quickly enough to be of use. Furthermore, a recent study (5) has revealed that the total number of *V. parahaemolyticus* cells does not appear to correlate directly with the number (or the presence of) TDH-producing *V. parahaemolyticus*. As an alternative approach, several PCR-based methods targeting *tdh* and *trh* were developed for the more specific detection of the TDH- and TRH-producing *V. parahaemolyticus* in seafood (1, 18). Nevertheless these PCR assays do not distinguish between DNA derived from viable or dead cells. This potentially leads to false-positive results for food samples where intact DNA sequences of *tdh* or *trh* are present despite the absence of TDH- and TRH-producing *V. parahaemolyticus* cells due to chemical or heat treatment. Similar technical difficulties in the differentiation between viable and dead cells in DNA-targeted diagnostics have long been discussed for other bacterial species (10, 12, 14).

In order to circumvent these technical obstacles, we describe here a novel combined culture and PCR method for the specific detection of viable TDH- or TRH-producing *V. parahaemolyticus* in seafood. The method employs two principals: (i) that only viable *V. parahaemolyticus* cells can penetrate through a soft-agar-coated filter due to their motility, and (ii) that DNAs released from ruptured virulent *V. parahaemolyticus* cells into enrichment medium can be eliminated by DNase pretreatment so that they do not interfere with subsequent multiplex PCR. With this method, the possible contamination of viable TDH- or TRH-producing *V. parahaemolyticus* in various seafood samples can be determined within 2 working days.

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MATERIALS AND METHODS

Bacterial strains. A total of 54 *V. parahaemolyticus* strains including two *tdh*-positive and *trh*-positive (tdh^+ trh^+) strains, 35 only *tdh*-positive strains, 9 only *trh*-positive strains, and 8 *tdh*-negative and *trh*-negative strains of various serotypes (i.e., O1:K1, O1:K25, O3:K6, O3:K48, O4:K6, O4:K8, O4:K68, and O8:K41) were used to evaluate the specificity of the multiplex PCR assay described below. Among them, *V. parahaemolyticus* KE 10540 (a clinical isolate; serotype O3:K46, tdh^+ trh^+) was used to determine the sensitivity of the multiplex PCR assay. Strains KE 10540, KE 10460 (an environmental isolate; O3:K56, lacking both *tdh* and *trh*) and an environmental isolate of *V. alginolyticus* AKO18 (motile) were used for the preliminary experiments described below. Furthermore, 23 strains of various serotypes positive for either or both *tdh* and *trh* were used for a subsequent quasi-field experiment. The strains were maintained on heart infusion agar (Difco Laboratories, Detroit, Mich.) containing 2% NaCl (final concentration) until use.

Specificity and sensitivity tests of multiplex PCR. Whole genomic DNAs of the 54 *V. parahaemolyticus* strains were prepared in Tris-EDTA buffer (pH 8.0) essentially as described elsewhere (1). The DNA preparations thus obtained were used as templates to evaluate the specificity of the multiplex PCR assay targeting both *tdh* and *trh*. KE 10540 was inoculated into alkaline peptone water (APW; 10 g of peptone and 10 g of NaCl in 1,000 ml of distilled water, pH 8.8) and incubated at 37°C with shaking for 10 h to obtain exponential growth (ca. 3.0×10^8 CFU/ml). After incubation, 100 μl each of a series of 10-fold dilutions (10^{-1} to 10^{-8} or ca. 3.0×10^7 to 30×10^7 CFU/ml) of the culture was dispensed into microtubes and heated at 100°C for 10 min. After centrifugation at $10,000 \times g$ for 5 min, the supernatants were used as template DNAs to determine the sensitivity of the multiplex PCR assay.

Multiplex PCR was performed with the oligonucleotide primers 5′-CCACTA CCACTCTCATATGC-3′ (sense primer) and 5′-GGTACTAAATGGCTGACA TC-3′ (antisense primer) at positions 451 to 469 and 713 to 694 in *tdh*, respectively (positions according to Honda et al. [6]) (GenBank accession no. D90238) to yield a 251-bp fragment and with the primers 5′-TTGGCTTCGATATTTTC AGTATCT-3′ (sense primer) and 5′-CATAACAAACATATGCCCATTTCC G-3′ (antisense primer) at positions 561 to 576 and 1211 to 1196 in *trh*, respectively (positions according to Lin et al. [11]) (GenBank accession no. L11929) to yield a 500-bp fragment. PCR amplification was performed in a total volume of 20 μl. Two microliters of each template DNA preparation was added to the PCR master mix, which consisted of 2 μl of 10× PCR buffer (Mg^{2+} free; Promega Corporation, Madison, WI), 2.4 μl of 25 mM $MgCl_2$ (final concentration, 3.0 mM), 0.25 μl of a deoxynucleoside triphosphate mixture (a 0.125 mM concentration of each deoxynucleoside triphosphate), 0.125 μl of each primer (0.125 μM concentration of each primer), and 0.125 μl (0.625 U) of *Taq* DNA polymerase (Takara Bio Co., Shiga, Japan), with the remaining volume consisting of distilled water. A GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, Calif.) was used for PCR amplification consisting of initial denaturation at 94°C for 3 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 60 s; and a final extension at 72°C for 5 min. Five microliters of the PCR products was electrophoresed on 2% agarose gels, stained with ethidium bromide (0.25 μg/ml), and photographed under UV light.

Preliminary experiments. In order to establish a reliable protocol for the method, the influence of incidental dead cells or viable avirulent strains and the effects of DNase treatment on the culture prior to DNA template preparation were evaluated as follows.

Preparation of bacterial inoculants. KE 10540 (tdh^+ trh^+) was inoculated to 200 ml of APW and incubated at 37°C with shaking for 10 h. After incubation, a portion of the culture was diluted with sterile saline to prepare a bacterial suspension of ca. 5.0×10^2 CFU/ml to be used as "live inoculant" of KE 10540. Another portion (100 ml) of the culture was centrifuged at $8,000 \times g$ for 10 min, and the supernatants were discarded. The bacterial pellet was resuspended in 10 ml of saline containing 200 ppm sodium hypochlorite and incubated at room temperature for 4 h to kill all viable cells. Subsequently, the bacterial cells were washed three times with sterile saline and finally suspended in 1 ml of sterile saline to be used as "dead inoculant" of KE 10540 (ca. 3.0×10^{10} dead cells/ml). In addition, KE 10460 (lacking both *tdh* and *trh*) or *V. alginolyticus* AKO 18 (lacking both *tdh* and *trh*) was inoculated into 100 ml of APW and incubated at 37°C with shaking for 10 h. After incubation, the culture was centrifuged at $8,000 \times g$ for 10 min. Bacterial cells were washed three times with sterile saline and finally suspended in 1 ml of sterile saline to be used as "live inoculant" (approximately 3.0×10^{10} CFU/ml) of KE 10460 or AKO 18.

Experiment 1a. Filter papers (Whatman no. 6; pore size, 3 μm; diameter, 110 mm [Maidstone, England]) folded into a cone shape were autoclaved. After autoclaving, the sterile filter papers were submerged for 5 min in APW containing 0.5% agar (agar no. 1; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) maintained at 50 to 60°C. After submersion, the filter papers (Fig. 1a) were removed from the medium and placed in an upright position on a sterile dish in a clean bench for 30 min to produce soft-agar-coated filters.

Two hundred microliters of viable inoculant of KE 10540 (tdh^+ trh^+) was added to APW (190 ml) in a sterile plastic container (BC2200; outside diameter, 73 mm; internal diameter, 63 mm; length, 90.5 mm; equipped with a screw lid) (Eiken Kizai Co. Ltd., Tokyo, Japan) in which the total cell number in the medium was calculated to be ca. 100 viable cells. A sterile polypropylene funnel (NL4252-0065; maximum diameter, 65 mm; minimum diameter, 16 mm; length, 67 mm) (Nalgene Co., Rochester, N.Y.) was placed over the container (Fig. 1b). The soft-agar-coated filter paper was then fitted onto the funnel, and 10 ml of APW was added into the cone (Fig. 1c); finally, a lid was loosely fitted over the container (Fig. 1d). The containers were then incubated at 37°C for 6, 8, 12, 16, or 20 h.

After incubation, 1 ml of the culture in the cone was transferred to a microtube and centrifuged at $10,000 \times g$ for 5 min. After centrifugation, the supernatant was discarded, and the bacterial pellet was suspended in 100 μl of sterile saline. The suspension was placed in another microtube and mixed with 26 μl of DNase solution containing 25 U of DNase (DNase I from bovine pancreas in 50% glycerol solution with 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM $CaCl_2$; Sigma Chemical Co., St. Louis, Mo.) and 14 μl of buffer solution (400 mM Tris-HCl, pH 7.5, 80 mM $MgCl_2$, 50 mM dithiothreitol in ultra-purified water) and incubated at 37°C for 1 h.

After DNase treatment, half (50 μl) of the mixture was placed in a microtube and heated at 100°C for 10 min and used as a source of template DNA for the multiplex PCR assay. In addition, we performed the following PCR assays on the template DNA in order to ensure the validity of any negative result of the multiplex PCR assay: PCR assay targeting the 16S rRNA sequence universal to nearly any bacterium with the oligonucleotide primers 5′-CAGGCCTAACAC ATGCAAGTC-3′ (sense primer) and 5′-GGGCGGWGTGTACAAGGC-3′ (antisense primer) to yield a ca. 1,300-bp fragment (13), and an assay targeting the ToxR gene sequence species-specific to *V. parahaemolyticus* with the oligonucleotide primers 5′-GTCTTCTGACGCAATCGTTG-3′ (sense primer) and 5′-ATACGAGTGGTTGCTGTCATG-3′ (antisense primer) to yield a 368-bp fragment (9) in order to confirm the presence of any bacterial cells and *V. parahaemolyticus* cells in the culture, respectively (collectively referred to as "positive control" PCR assays hereafter).

Meanwhile, the remaining mixture (50 μl) was placed in a microtube and centrifuged at $10,000 \times g$ for 5 min. After centrifugation, 20 μl of the supernatant was placed in a microtube and heated at 100°C for 10 min and then used as a source of "template DNA" for the multiplex PCR assay in order to confirm that any free-ranging DNA fragments containing *tdh* or *trh* had been completely digested through DNase treatment (referred to as "negative control" PCR assay hereafter).

Experiment 1b. Two hundred microliters of viable inoculant of KE 10540 or 200 μl of the dead inoculant of KE 10540 was added to APW (190 ml) in a sterile plastic container, in which the total number of cells in the medium was calculated to be ca. 100 viable cells or ca. 6.0×10^8 dead cells, respectively. A soft-agar-coated filter paper with a sterile funnel was placed over the container. Ten milliliters of APW was then added to the bottom of the cone, and finally the lid was loosely fitted over the container. The container was then incubated at 37°C for 20 h. After incubation, the culture was treated with or without DNase, and template DNAs thus prepared were used for the multiplex PCR assay and the positive and negative control PCR assays.

Experiment 2a. Two hundred microliters of the dead inoculant of KE 10540 and 200 μl of the viable inoculant of KE 10460 or AKO 18 (combinations 1 and 2, respectively) were added to APW (190 ml) in a sterile plastic container, in which the initial cell number in the medium was calculated to be ca. 6.0×10^8 dead cells for KE 10540 and ca. 6.0×10^8 viable cells for KE 10460 or AKO 18. As described for experiment 1, a sterile funnel with a cone-shaped filter paper coated with 0.5% soft agar was placed over the container, and 10 ml of APW was added to the bottom of the cone; finally, a lid was loosely fitted over the container. The container was then incubated at 37°C for 20 h. After incubation, template DNA from the culture that had been pretreated with DNase was assayed by the multiplex PCR and the positive and negative control PCR assays. The experiment was repeated in triplicate.

Experiment 2b. Two hundred microliters of the viable inoculant of KE 10540 and 200 μl of the live inoculant of KE 10460 or AKO 18 (combinations 3 and 4, respectively) were added to APW (190 ml) in a sterile plastic container, in which the initial cell number in the medium was calculated to be ca. 100 viable cells of KE 10540 and ca. 6.0×10^8 viable cells of KE 10460 or AKO 18. The samples

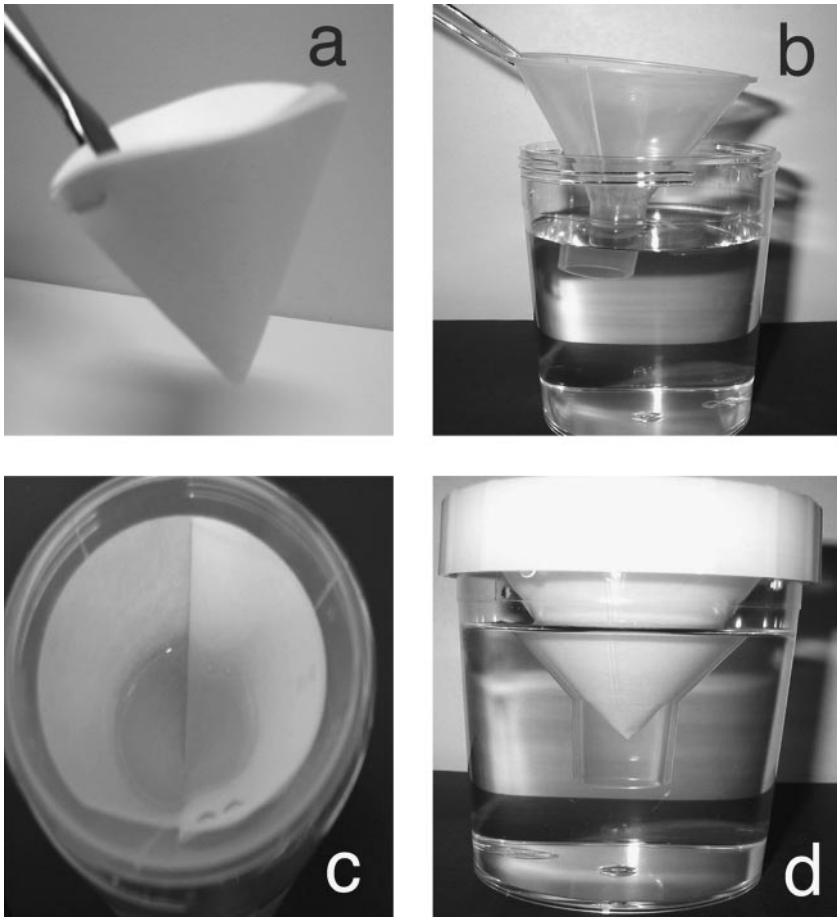

FIG. 1. Photographs of the soft-agar-coated filter method. (a) A Whatman no. 6 filter paper is folded into a cone shape and coated with 0.5% agar. (b) A sterile polypropylene funnel is placed over an alkaline peptone water container. (c) Ten milliliters of APW is added to the cone. (d) A lid is loosely fitted over the container.

thus prepared were then processed and assayed in the same manner as described for experiment 2a. The experiment was repeated in triplicate.

Quasi-field experiment. Each of the 23 strains of the various serotypes (see Table 2) that were positive for either or both *tdh* and *trh* was inoculated into 100 ml of APW and incubated at 37°C with shaking for 10 h. After incubation, a portion of the culture was diluted with sterile saline to prepare a bacterial suspension of ca. 1×10^2 to $\sim 2 \times 10^2$ CFU/ml. Twenty grams of various commercially available seafood (seven packages of prawns, four packages of short-necked clams, five packages of scallops, and five packages of oysters) with or without 200 μl of the above bacterial suspension was added to APW (170 ml) in a sterile plastic container in which the initial number of artificially inoculated viable cells per 1 g of each seafood sample was calculated to be approximately 5 to ~10. The samples thus prepared were then processed and incubated in the same manner as described for experiment 2a.

After incubation, 1 ml of the culture in the cone was transferred to a microtube and centrifuged at 10,000 × *g* for 5 min, and the bacterial pellet was suspended in 100 μl of sterile saline. The suspension was processed in the same manner described above to prepare sources of template DNAs to be analyzed by multiplex PCR and the positive and negative control PCR assays.

RESULTS

Specificity and sensitivity of the multiplex PCR assay. Assay results of multiplex PCR on the 54 *V. parahaemolyticus* strains were consistent with those obtained from the conventional *tdh*- or *trh*-targeted PCR assay (19). The sensitivity of multiplex PCR was determined using serial 10-fold dilutions of KE 10540 cells. Following agarose gel electrophoresis, the minimal cell concentration detectable was 3.0×10^4 cells/ml (data not shown). The corresponding sensitivity was ca. 60 DNA copies in 2 μl of template preparation.

Incubation time and DNase pretreatment. The results of experiment 1a indicated that the reliable incubation time required for detecting the inoculated viable strain positive for both *tdh* and *trh* was 20 h (data not shown). The results of experiment 1b indicated that DNase pretreatment did not adversely affect the multiplex PCR assay on viable cells (Fig. 2). The assay without DNase pretreatment on the sample containing dead virulent cells yielded PCR products of *tdh* and *trh*, indicating that DNA released from dead cells into the medium had indeed diffused through the soft-agar-coated filter to the medium inside the cone. Meanwhile, the multiplex PCR assay and the negative PCR assay on the DNase-pretreated sample yielded no PCR products, indicating that the "diffused-through" DNAs were completely digested, thereby preventing any false-positive assay result.

Influences of competing bacterial cells. Results of experiment 2a (Table 1) indicated that the sample initially containing dense populations of dead tdh^+ trh^+ cells and viable cells lacking *tdh* and *trh* (combinations 1 and 2) did not yield any

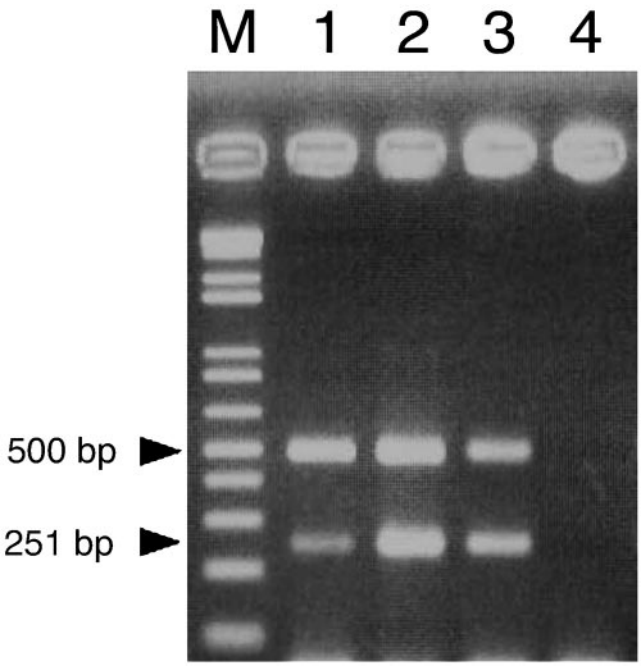

FIG. 2. Results of the multiplex PCR assay with or without DNase pretreatment of cultures initially inoculated with viable or dead *V. parahaemolyticus* KE 10540 (*tdh*$^+$ *trh*$^+$) in the soft-agar-coated filter method. Template DNAs were prepared from the original culture initially inoculated with viable cells (lane 1); the same culture was also treated with DNase (lane 2). Template DNAs were prepared from the original culture initially inoculated with dead cells (lane 3); the same culture was also treated with DNase (lane 4). Lane M, molecular size markers (mixture of phage λ DNA digested with HindIII and phage ϕX174 DNA digested with HaeIII). PCR amplicon sizes for *tdh* and *trh* are 251 bp and 500 bp, respectively.

positive results in the multiplex PCR assay, indicating that dead cells (without motility) of the *tdh*$^+$ *trh*$^+$ *V. parahaemolyticus* did not pass through the soft-agar-coated filter. In contrast, the results of experiment 2b (Table 1) indicated that the sample initially containing a very small population of viable virulent cells and a dense population of viable avirulent cells (combinations 3 and 4) yielded positive results in the multiplex PCR assay. This, in turn, indicates that concomitant bacterial cells, even densely populated, in the sample do not prevent the passage of motile, viable virulent *V. parahaemolyticus* cells through the filter.

Results of the quasi-field experiment. The seafood samples artificially inoculated with small numbers of viable virulent strains of various serotypes all yielded positive results in the subsequent multiplex PCR assay, whereas those without artificial inoculation did not (Table 2).

DISCUSSION

Velammal et al. (20) claimed that TDH-producing *V. parahaemolyticus* grows selectively in a gastropod species inhabiting an estuarine environment in Japan, suggesting that gastropods are a natural reservoir. Cook et al. (3) also reported that TDH-producing *V. parahaemolyticus* was detected in some Atlantic and Gulf Coast molluscan shellfish, possibly due to the uneven distribution of the organism in the environment. Furthermore, Hara-Kudo et al. (5) reported that the total numbers of *V. parahaemolyticus* cells did not reflect that of TDH-producing strains in seafood. The evidence suggests the development of a more sensitive method for detecting the toxin-producing *V. parahaemolyticus*, regardless of the numbers of the avirulent background cells of *V. parahaemolyticus* in seafood.

In this context, many PCR-based techniques (1, 8, 18) targeting *tdh* and *trh* have been developed so far to improve the detection level of the pathogen in various types of seafood. One challenge presented by PCR-based methods is the interference of *tdh* or *trh* derived from dead cells present in seafood. In seafood processing, sanitation is undertaken frequently using heat or chlorine-based disinfectants in the form of sodium and calcium hypochlorite (15). The procedure kills pathogens in seafood completely but may leave intact DNA including *tdh* or *trh*, thereby yielding false-positive results in a subsequent PCR-based assay. Furthermore, inadequate heat or chemical killing may leave a few viable virulent cells with a large background of dead virulent cells in the seafood, yielding the same positive results in the assay. These situations make it difficult to

TABLE 1. Results of the multiplex PCR assay and the positive and negative control PCR assays on the cultures obtained from inside the soft-agar-coated filter after enrichment and inoculation with combinations of viable or dead cells of *V. parahaemolyticus* or *V. algninolyticus*

Combination no.	Strain and serotype	Cell type	No. of cells	Results of the PCR assays on cultures in the soft-agar-coated filter after enrichment for 20 h[a]														
				Test 1					Test 2					Test 3				
				tdh	*trh*	16S	*toxR*	NC	*tdh*	*trh*	16S	*toxR*	NC	*tdh*	*trh*	16S	*toxR*	NC
1	*V. parahaemolyticus* KE 10540 (*tdh*$^+$ *trh*$^+$) *V. parahaemolyticus* KE 10460[b]	Dead Viable	ca. 6.0×10^8 ca. 6.0×10^8	−	−	+	+	−	−	−	+	+	−	−	−	+	+	−
2	*V. parahaemolyticus* KE 10540 (*tdh*$^+$ *trh*$^+$) *V. alginolyticus* AKO 18	Dead Viable	ca. 6.0×10^8 ca. 6.0×10^8	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−
3	*V. parahaemolyticus* KE 10540 (*tdh*$^+$ *trh*$^+$) *V. parahaemolyticus* KE 10460[b]	Viable Viable	ca. 100 ca. 6.0×10^8	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−
4	*V. parahaemolyticus* KE 10540 (*tdh*$^+$ *trh*$^+$) *V. alginolyticus* AKO 18	Viable Viable	ca. 100 ca. 6.0×10^8	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−

[a] The multiplex PCR assay for detection of *tdh* and *trh*, the positive control PCR assays for detection of universal bacterial 16S rRNA sequences (16S) and species-specific *toxR* sequence and the negative control PCR assay (NC). The enrichment medium was APW (100 ml).
[b] Strain is negative for both *tdh* and *trh*.

TABLE 2. Results of the multiplex PCR assay and the positive and negative control PCR assays by the soft-agar-coated filter method on seafood artificially inoculated with *V. parahaemolyticus*

Sample type	Sample no.	Origin or source (by sample group)	*V. parahaemolyticus* strain artificially inoculated to the sample (serotype)[a]	Results of the PCR assays on culture in the soft-agar-coated filter after enrichment for 20 h[b]				
				tdh	*trh*	16S	*toxR*	NC
Prawn	PR1	Domestic sea	KE 10457 (O3:K6)[c]	+	−	+	+	−
	PR1		KE 10540 (O3:K46)[d]	+	+	+	+	−
	PR1		None[f]	−	−	+	−	−
	PR2	Imported from Vietnam	KE 9984 (O3:K6)[e]	−	+	+	+	−
	PR2		None	−	−	+	+	−
	PR3	Domestic sea	KE 10540 (O3:K46)[d]	+	+	+	+	−
	PR3		None	−	−	+	−	−
	PR4	Imported from Vietnam	KE 10484 (O3:K6)[c]	+	−	+	+	−
	PR4		None	−	−	+	−	−
	PR5	Imported from Thailand	KE 10540 (O3:K46)[d]	+	+	+	+	−
	PR5		VP O12 (O12:K40)[e]	−	+	+	+	−
	PR5		None	−	−	+	+	−
	PR6	Imported from Malaysia	AQ4431 (O13:K72)[d]	+	+	+	+	−
	PR6		AN-2189 (O4:K68)[c]	+	−	+	+	−
	PR6		KIH VP24 (O3:K5)[e]	−	+	+	+	−
	PR6		None	−	−	+	+	−
	PR7	Domestic sea	KIH 03-57 (O4:K68)[c]	+	−	+	+	−
	PR7		KIH VP19 (O4:K4)[e]	−	+	+	+	−
	PR7		None	−	−	+	+	−
Short-necked clam	CL1	Domestic sea	KE 10540 (O3:K46)[d]	+	+	+	+	−
	CL1		None	−	−	+	−	−
	CL2	Domestic sea	NIID 956-98 (O3:K6)[c]	+	−	+	+	−
	CL2		None	−	−	+	−	−
	CL3	Domestic sea	KE 10443 (O3:K6)[e]	−	+	+	+	−
	CL3		None	−	−	+	+	−
	CL4	Imported from China	KIH VP8 (O1:KUT)[d]	+	+	+	+	−
	CL4		VP O2 (O2:K3)[c]	+	−	+	+	−
	CL4		None	−	−	+	−	−
Scallop	SC1	Domestic sea	VP O2 (O3:K6)[c]	+	−	+	+	−
	SC1		None	−	−	+	+	−
	SC2	Domestic sea	DMST17871 (O3:K6)[c]	+	−	+	+	−
	SC2		None	−	−	+	−	−
	SC3	Domestic sea	KE 10443 (O3:K6)[e]	−	+	+	+	−
	SC3		None	−	−	+	+	−
	SC4	Domestic sea	VP K18 (O6:K18)[d]	+	+	+	+	−
	SC4		KE 10538 (O4:K8)[c]	+	−	+	+	−
	SC4		None	−	−	+	−	−
	SC5	Domestic sea	AQ4431 (O13:K72)[d]	+	+	+	+	−
	SC5		KIH VP19 (O4:K4)[e]	−	+	+	+	−
	SC5		None	−	−	+	−	−
Oyster	OY1	Domestic sea	KE 10540 (O3:K46)[d]	+	+	+	+	−
	OY1		None	−	−	+	+	−
	OY2	Domestic sea	AP18000 (O1:K25)[c]	+	−	+	+	−
	OY2		KE 10540 (O3:K46)[d]	+	+	+	+	−
	OY2		KE 10443 (O3:K6)[e]	−	+	+	+	−
	OY2		None	−	−	+	+	−
	OY3	Domestic sea	KE 10541 (O8:K41)[c]	+	−	+	+	−
	OY3		TVP 1894 (O3:K6)[e]	−	+	+	+	−
	OY3		None	−	−	+	−	−
	OY4	Domestic sea	KE 10540 (O3:K46)[d]	+	+	+	+	−
	OY4		None	−	−	+	+	−
	OY5	Domestic sea	KIH 03-57 (O4:K68)[c]	+	−	+	+	−
	OY5		KIH VP19 (O4:K4)[d]	−	+	+	+	−
	OY5		None	−	−	+	−	−

[a] Initial concentration of the inoculum was 5 to 10 viable cells per gram of sample.
[b] PCR assays include the multiplex PCR for detection of *tdh* and *trh*, the positive control PCR assays for detection of universal 16S rRNA sequences (16S) and species-specific *toxR* sequence, and the negative control (NC) PCR assay.
[c] *tdh*-positive and *trh*-negative strain.
[d] *tdh*- and *trh*-positive strain.
[e] *tdh*-negative and *trh*-positive strain.
[f] None, not inoculated with any strain positive for either *tdh* or *trh* to make a tentatively "negative" control sample.

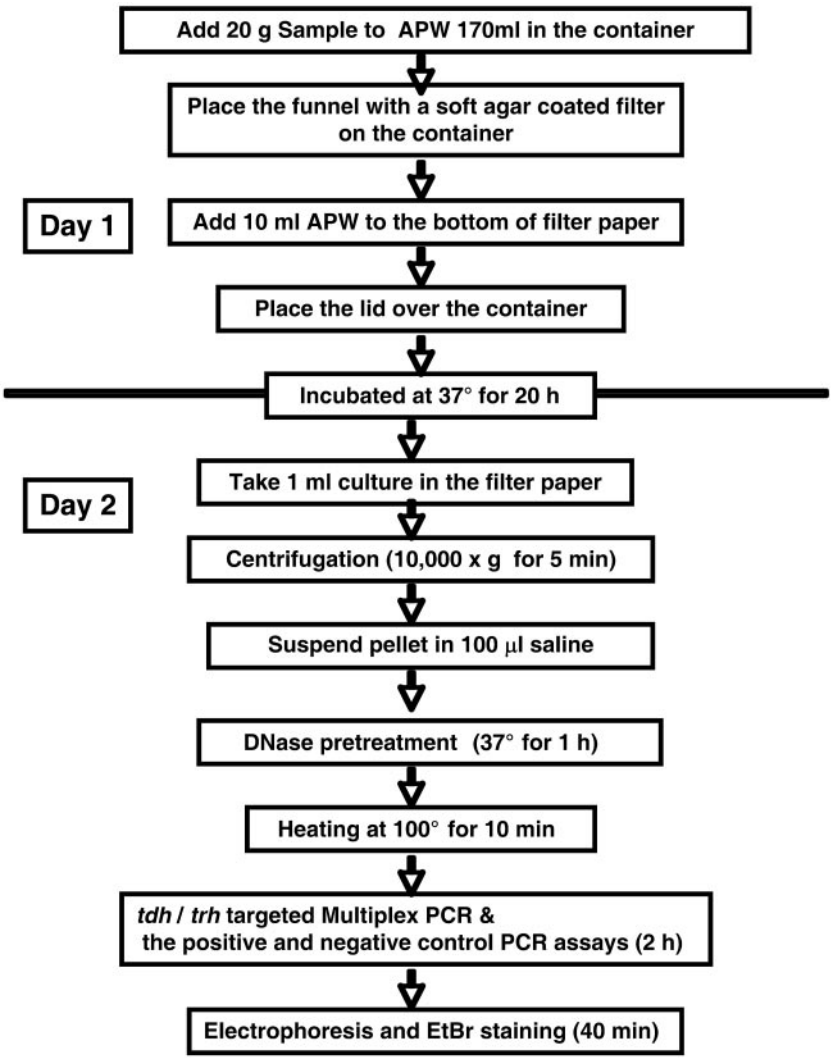

FIG. 3. Flow chart of novel detection system for viable *tdh*- and *trh*-positive *V. parahaemolyticus* in seafood with the soft-agar-coated filter method.

accurately determine the safety of seafood. Our soft-agar-coated filter method will circumvent these problems since the method detects viable motile cells, capable of penetrating soft-agar-coated filter paper and targeting *tdh* or *trh* derived from viable bacteria, thus avoiding false-positive results arising from the amplification of genes from DNA released from nonviable cells. With this method (summarized in Fig. 3), contamination levels of five viable cells of pathogenic *V. parahaemolyticus* per gram of a sample can be detected within two working days regardless of the background microflora. This will greatly improve the current labor-intensive, time-consuming safety testing procedures against pathogenic *V. parahaemolyticus* based on the conventional MPN method.

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