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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 thermo-food (2). Past epidemiological studies (6, 7, 16) revealed a strong association between gastroenteritis and both thermo-

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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:K1) were used to evaluate the specificity of the multiplex PCR assay described below. Among them, *V. parahaemolyticus* KE 10540 (a clinical isolate; serotype O3:K6, *tdh*-*trh*) was used to determine the specificity 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* AK01 (motile) were used for the preliminary experiments described below. Furthermore, 23 strains of various serotypes positive for either 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.

**Preparation of bacterial inoculants.** Whole genomic DNAs of the 54 *V. parahaemolyticus* strains were prepared in Trios-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 8 to 10 h to obtain exponential growth (ca. 3.0 × 10^7 to 3.0 × 10^8 CFU/ml). After incubation, 100 µl of a suspension of 10^8 viable cells of KE 10460 or AKO 18. The samples were then inoculated into 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 × 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 MgCl₂, Sigma Chemical Co., St. Louis, Mo.) and 14 µl of buffer solution (400 mM Tris-HCl, pH 7.5, 80 mM MgCl₂, 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 the following PCR experiments, the following primers were used 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 all bacteria with the oligonucleotide primers 5'-CCAGGCTTACACATGAACTG-3' (sense primer) and 5'-GGGCGGWGTGACAATGATCT-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'-TGTCCTGCAGCACAATGGT-3' (sense primer) and 5'-ATACGGTGTTCGTGCTATG-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 10,800 × 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 10460 and ca. 6.0 × 10^9 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 treated 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 × 10^7 viable cells of KE 10460 or AKO 18. The samples 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 submergence, 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.
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. $1 \times 10^7$ to $2 \times 10^7$ 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 g of each seafood sample was calculated to be approximately 5 to $10^8$. 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 $\times g$ 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 $3.0 \times 10^4$ 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
positive results in the multiplex PCR assay, indicating that
dead cells (without motility) of the \( \text{tdh}^+ \text{trh}^+ \) \( V. \) 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 \( \text{tdh} \) and \( \text{trh} \) 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 \( \text{tdh} \) or \( \text{trh} \) 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 \( \text{tdh} \) and \( \text{trh} \) derived from dead cells present in seafood. In 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

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**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 \( V. \) alginolyticus**

<table>
<thead>
<tr>
<th>Combination no.</th>
<th>Strain and serotype</th>
<th>Cell type</th>
<th>No. of cells</th>
<th>Results of the PCR assays on cultures in the soft-agar-coated filter after enrichment for 20 h*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( V. ) parahaemolyticus KE 10540 ( (\text{tdh}^+ \text{trh}^+) ) ( V. ) parahaemolyticus KE 10460a</td>
<td>Dead</td>
<td>ca. ( 6.0 \times 10^6 )</td>
<td>( \text{tdh} ) ( \text{trh} ) 16S toxR NC ( \text{tdh} ) ( \text{trh} ) 16S toxR NC</td>
</tr>
<tr>
<td>2</td>
<td>( V. ) parahaemolyticus KE 10540 ( (\text{tdh}^+ \text{trh}^+) ) ( V. ) alginolyticus AKO 18</td>
<td>Dead</td>
<td>ca. ( 6.0 \times 10^6 )</td>
<td>( \text{tdh} ) ( \text{trh} ) 16S toxR NC ( \text{tdh} ) ( \text{trh} ) 16S toxR NC</td>
</tr>
<tr>
<td>3</td>
<td>( V. ) parahaemolyticus KE 10540 ( (\text{tdh}^+ \text{trh}^+) ) ( V. ) parahaemolyticus KE 10460a</td>
<td>Viable</td>
<td>ca. 100</td>
<td>( \text{tdh} ) ( \text{trh} ) 16S toxR NC ( \text{tdh} ) ( \text{trh} ) 16S toxR NC</td>
</tr>
<tr>
<td>4</td>
<td>( V. ) alginolyticus KE 10540 ( (\text{tdh}^+ \text{trh}^+) ) ( V. ) alginolyticus AKO 18</td>
<td>Viable</td>
<td>ca. ( 6.0 \times 10^6 )</td>
<td>( \text{tdh} ) ( \text{trh} ) 16S toxR NC ( \text{tdh} ) ( \text{trh} ) 16S toxR NC</td>
</tr>
</tbody>
</table>

a The multiplex PCR assay for detection of \( \text{tdh} \) and \( \text{trh} \), 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 \( \text{tdh} \) and \( \text{trh} \).
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*

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Sample no.</th>
<th>Origin or source (by sample group)</th>
<th><em>V. parahaemolyticus</em> strain artificially inoculated to the sample (serotype)*</th>
<th>Results of the PCR assays on culture in the soft-agar-coated filter after enrichment for 20 h&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prawn</td>
<td>PR1</td>
<td>Domestic sea</td>
<td>KE 10457 (O3:K6)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ - + + -</td>
</tr>
<tr>
<td></td>
<td>PR1</td>
<td></td>
<td>KE 10540 (O3:K46)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
</tr>
<tr>
<td></td>
<td>PR1</td>
<td></td>
<td>None&lt;sup&gt;i&lt;/sup&gt;</td>
<td>- - - + -</td>
</tr>
<tr>
<td></td>
<td>PR2</td>
<td>Imported from Vietnam</td>
<td>KE 9984 (O3:K6)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>- + + + -</td>
</tr>
<tr>
<td></td>
<td>PR3</td>
<td>Domestic sea</td>
<td>KE 10540 (O3:K46)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
</tr>
<tr>
<td></td>
<td>PR3</td>
<td></td>
<td>None</td>
<td>- - + - -</td>
</tr>
<tr>
<td></td>
<td>PR4</td>
<td>Imported from Vietnam</td>
<td>KE 10484 (O3:K6)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ - + + -</td>
</tr>
<tr>
<td></td>
<td>PR4</td>
<td></td>
<td>None</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>PR5</td>
<td>Imported from Thailand</td>
<td>KE 10540 (O3:K46)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
</tr>
<tr>
<td></td>
<td>PR5</td>
<td></td>
<td>VP O12 (O12:K40)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
</tr>
<tr>
<td></td>
<td>PR6</td>
<td>Imported from Malaysia</td>
<td>AQ431 (O13:K72)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
</tr>
<tr>
<td></td>
<td>PR6</td>
<td></td>
<td>AN-2189 (O4:K68)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ - + + -</td>
</tr>
<tr>
<td></td>
<td>PR6</td>
<td></td>
<td>KIH VP24 (O3:K5)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>- + + + -</td>
</tr>
<tr>
<td></td>
<td>PR6</td>
<td></td>
<td>None</td>
<td>- - + + -</td>
</tr>
<tr>
<td></td>
<td>PR7</td>
<td>Domestic sea</td>
<td>KIH 03-57 (O4:K68)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ - + + -</td>
</tr>
<tr>
<td></td>
<td>PR7</td>
<td></td>
<td>KIH VP19 (O4:K4)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>- + + + -</td>
</tr>
<tr>
<td></td>
<td>PR7</td>
<td></td>
<td>None</td>
<td>- + + + -</td>
</tr>
<tr>
<td>Short-necked clam</td>
<td>CL1</td>
<td>Domestic sea</td>
<td>KE 10540 (O3:K46)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
</tr>
<tr>
<td></td>
<td>CL1</td>
<td></td>
<td>None</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>CL2</td>
<td>Domestic sea</td>
<td>NIIID 956-98 (O3:K6)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ - + + -</td>
</tr>
<tr>
<td></td>
<td>CL2</td>
<td></td>
<td>None</td>
<td>- - - - -</td>
</tr>
<tr>
<td></td>
<td>CL3</td>
<td>Domestic sea</td>
<td>KE 10443 (O3:K6)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
</tr>
<tr>
<td></td>
<td>CL3</td>
<td></td>
<td>None</td>
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<td></td>
<td>CL4</td>
<td>Imported from China</td>
<td>KIH VP8 (O1:KUT)&lt;sup&gt;cf&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>CL4</td>
<td></td>
<td>VP O2 (O2:K3)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ - + + -</td>
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<tr>
<td></td>
<td>CL4</td>
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<tr>
<td>Scallop</td>
<td>SC1</td>
<td>Domestic sea</td>
<td>VP O2 (O3:K6)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ - + + -</td>
</tr>
<tr>
<td></td>
<td>SC1</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>SC2</td>
<td>Domestic sea</td>
<td>DMST17871 (O3:K6)&lt;sup&gt;cf&lt;/sup&gt;</td>
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<td></td>
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<td>SC3</td>
<td>Domestic sea</td>
<td>KE 10443 (O3:K6)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>- + + + -</td>
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<td></td>
<td>SC3</td>
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<td>None</td>
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<tr>
<td></td>
<td>SC4</td>
<td>Domestic sea</td>
<td>VP K18 (O6:K18)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
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<tr>
<td></td>
<td>SC4</td>
<td></td>
<td>KE 10538 (O4:K8)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + -</td>
</tr>
<tr>
<td></td>
<td>SC4</td>
<td></td>
<td>None</td>
<td>- + + + -</td>
</tr>
<tr>
<td></td>
<td>SC5</td>
<td>Domestic sea</td>
<td>AQ431 (O13:K72)&lt;sup&gt;cf&lt;/sup&gt;</td>
<td>+ + + + +</td>
</tr>
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<td>SC5</td>
<td></td>
<td>KIH VP19 (O4:K4)&lt;sup&gt;cf&lt;/sup&gt;</td>
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<td>OY1</td>
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<td>KE 10540 (O3:K46)&lt;sup&gt;cf&lt;/sup&gt;</td>
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<td>AP18000 (O1:K25)&lt;sup&gt;cf&lt;/sup&gt;</td>
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<sup>a</sup> Initial concentration of the inoculum was 5 to 10 viable cells per gram of sample.

<sup>b</sup> 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.

<sup>c</sup> *tdh*-positive and *trh*-negative strain.

<sup>d</sup> *tdh*-negative and *trh*-positive strain.

<sup>e</sup> *tdh*-positive and *trh*-positive strain.

<sup>f</sup> None, not inoculated with any strain positive for either *tdh* or *trh* to make a tentatively “negative” control sample.
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.

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.

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REFERENCES


