Evaluation of a Tissue Culture-Based Approach for Differentiating between Virulent and Avirulent Vibrio parahaemolyticus Strains Based on Cytotoxicity

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ABSTRACT

The ability of only a subset of Vibrio parahaemolyticus strains to cause human infection underscores the need for an analytical method that can effectively differentiate between pathogenic strains and those that do not cause disease. We tested the feasibility of a tissue culture–based assay to determine whether clinical isolates could be differentiated from nonclinical isolates based on relative isolate cytotoxicity. To screen for cytotoxic capability, we measured relative extracellular lactate dehydrogenase as an indicator of host cell damage in five different mammalian cell lines in the presence of V. parahaemolyticus. Isolates originating from clinical sources exhibited 15.5 to 59.3% relative cytotoxicity, whereas those originating from food sources exhibited 4.4 to 54.9% relative cytotoxicity. In the presence of ~1.2 × 10^6 cells, cytotoxicity was 1.6- to 3.5-fold higher (P < 0.05) for clinical isolates than for nonclinical isolates in L2, Henle 407, and Caco-2 cell lines. V. parahaemolyticus serotype O3:K6 clinical isolates had 1.6- to 2.1-fold higher cytotoxicity than did the non-O3:K6 clinical isolates, with significantly higher cytotoxicity in HeLa, J774A.1, and Henle 407 cells than in L2 and Caco-2 cells. Because V. parahaemolyticus often is found in oysters, the effect of the presence of an oyster matrix on assay efficacy was also tested with L2 cells. The cytotoxicity elicited by a highly cytotoxic V. parahaemolyticus isolate was not affected by the presence of oyster tissue, suggesting that an oyster matrix will not interfere with assay sensitivity. In the present format, this assay can detect the presence of >10^5 cells of a virulent V. parahaemolyticus strain in an oyster matrix.

As a natural inhabitant of estuarine marine water, Vibrio parahaemolyticus is widely distributed in inshore marine waters throughout the world. Some V. parahaemolyticus strains are capable of causing human illness, and a subset of these strains has been implicated in outbreaks of foodborne disease. No single genetic determinant has been identified as both necessary and sufficient for V. parahaemolyticus virulence, although many factors have been proposed to be involved (45). Thermostable direct hemolysin (TDH) is one factor that has long been considered a major V. parahaemolyticus virulence marker (29, 32). A V. parahaemolyticus strain bearing a loss-of-function mutation in the tdh gene has attenuated virulence in the rabbit ileal loop model (32). However, loss of TDH did not impair V. parahaemolyticus virulence in all model systems (9, 11), indicating that TDH is not the sole contributor to V. parahaemolyticus pathogenicity. A TDH-related hemolysin (TRH) also has been linked to V. parahaemolyticus gastroenteritis (16, 37, 38). However, V. parahaemolyticus strains lacking TDH have been associated with foodborne disease illnesses (10), and some clinical isolates possess neither tdh nor trh (23, 26). Other putative factors associated with V. parahaemolyticus pathogenesis include urea hydrolysis, the production of vibrioferrin, and the presence of a type III secretion system (14, 24, 44). Currently, standard methods for isolation and differentiation of V. parahaemolyticus dictate that isolates determined to be tdh positive by PCR or colony hybridization (40) are potential human pathogens. However, these conventional detection strategies that specifically detect tdh alone may fail to recognize potentially virulent tdh-negative V. parahaemolyticus strains.

Because V. parahaemolyticus strains can exchange genetic material (24) and hence genetic markers (1, 33), we hypothesized that measurement of V. parahaemolyticus virulence-associated characteristics is a more reliable and robust approach for detection of pathogenic V. parahaemolyticus strains than is the presence or absence of specific genetic markers. In support of this concept, previous work demonstrated the potential of tissue culture–based assays for detection of cytotoxic effects of pathogenic Vibrio strains (8). For example, V. parahaemolyticus serotype O3:K6 strains have a clearly detectable cytopathogenic phenotype in human epithelial HeLa cells (47).

A cytotoxicity assay was developed to measure release of lactate dehydrogenase (LDH) from mammalian cells as an indicator of cell damage in the presence of V. parahaemolyticus. The objectives of this study were to (i) quantify the relative cytotoxicity of various V. parahaemolyticus isolates from food or clinical sources in multiple tissue cell lines representing different host cell types and (ii) assess whether a tissue culture–based method could be used to detect the presence of pathogenic V. parahaemolyticus present in a seafood matrix.

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TABLE 1. Vibrio parahaemolyticus isolates used in this study

| Laboratory no. | FDA identification | Serotype | Source | Place of isolation | Year of isolation | tdf PCR
|---------------|--------------------|----------|--------|-------------------|------------------|---------
| FSL-Y1-001    | 92000713 (1)       | O8       | Food (clam) | Unknown           | 1992             | -       |
| FSL-Y1-002    | NY477              | O4:K8    | Food (oyster) | New York          | 1977             | +       |
| FSL-Y1-003    | T3980              | O4:K13   | Clinical    | Japan             | Unknown          | +       |
| FSL-Y1-004    | CRAB               | Unknown  | Food      | Washington        | 1972             | -       |
| FSL-Y1-005a   | 5C-1C              | O1       | Food (oyster) | Washington        | 1988             | -       |
| FSL-Y1-006    | M350A              | O5       | Food (oyster) | Washington        | 1994             | -       |
| FSL-Y1-010a   | T-3979             | O5:K15   | Clinical    | Japan             | Unknown          | +       |
| FSL-Y1-011a   | 8332924            | O1:K56   | Food (oyster) | Gulf             | 1983             | -       |
| FSL-Y1-012    | 48432              | O4:K12   | Clinical    | Washington        | 1991             | -       |
| FSL-Y1-013    | 47978              | O6:K18   | Clinical    | Washington        | 1991             | -       |
| FSL-Y1-014a   | AN-5034            | O4:K6c   | Clinical    | Bangladesh        | 1996             | +       |
| FSL-Y1-015    | AN-16000           | O1:KUTcd | Clinical    | Bangladesh        | 1998             | +       |
| FSL-Y1-016    | TX-2103            | O3:K6    | Clinical    | Texas             | 1998             | +       |
| FSL-Y1-017b   | NY-3064            | O3:K6    | Clinical    | New York          | 1998             | +       |
| FSL-Y1-021b   | U-5474             | Old 03:K6e | Clinical    | Bangladesh        | 1980             | +       |
| FSL-Y1-023b   | VP86               | O3:K6    | Clinical    | Calcutta          | 1996             | +       |
| FSL-Y1-024b   | VP199              | O3:K6    | Clinical    | Calcutta          | 1997             | +       |
| FSL-Y1-025    | VP208              | O3:K6    | Clinical    | Calcutta          | 1997             | +       |
| FSL-Y1-026b   | VP155              | O3:K6    | Clinical    | Calcutta          | 1996             | +       |
| FSL-Y1-036    | FIHES98V1-32-4     | O3:K6    | Clinical    | Japan             | 1998             | +       |
| FSL-Y1-046    | BAC-98-03255       | O3:K6    | Clinical    | New York          | Unknown          | -       |
| FSL-Y1-059    | 98-792-807 (27)    | O8:K74   | Food (oyster) | Galveston Bay    | 1998             | -       |
| FSL-Y1-068    | DI-A6-031699       | O4:K9    | Food (oyster) | Alabama          | Unknown          | -       |
| FSL-Y1-069    | DI-B11-031699      | O4:K22   | Food (oyster) | Alabama          | Unknown          | -       |
| FSL-Y1-073    | DI-F8-031699       | O11:KUTd | Food (oyster) | Alabama          | Unknown          | -       |
| FSL-Y1-081    | DI-A6-020800       | O11:K61  | Food (oyster) | Alabama          | Unknown          | -       |

a Results were compiled from a previous study (47) and from unpublished data.

b Isolates tested for CHO elongation activity.

c Strains deemed genetically similar to 03:K6 strains by arbitrarily primed PCR, ribotypes, and pulsed-field gel electrophoresis pattern analyses (5, 26).

d UT, untypeable.

O3:K6 strain isolated before 1996 that is genetically different from the O3:K6 strains isolated after 1996.

MATERIALS AND METHODS

Bacterial isolates. V. parahaemolyticus isolates used in this study were provided by the U.S. Food and Drug Administration (FDA) (Table 1). Serotypes were determined previously by the Centers for Disease Control and Prevention. Upon receipt, all isolates were streaked on tryptic soy agar (Difco, Becton Dickinson, Sparks, Md.) supplemented with 2% NaCl (TSAS) to obtain single colonies. Isolates were stored in tryptic soy broth supplemented with 2% NaCl (TSBS) and 20% glycerol at -80°C. Isolates tested for CHO elongation activity.

Clinical and Experimental Materials. Materials and Methods. The hemolytic activity of the isolates was determined on sheep blood agar plates (Difco, Becton Dickinson, Sparks, Md.) after incubation at 37°C for 18-24 h. The hemolytic activity was expressed as the percentage of hemolysis, which was calculated as follows:

\[
\text{Hemolysis} = \frac{A_{\text{sample}} - A_{\text{spontaneous}}}{A_{\text{total}} - A_{\text{spontaneous}}} \times 100\%
\]

where \(A_{\text{sample}}\) is the absorbance of infected tissue cells and \(A_{\text{spontaneous}}\) is the absorbance of uninfected tissue cells. To determine \(A_{\text{total}}\), the maximal release of LDH, tissue cells were lysed by adding 15 \(\mu \text{L}\) of lysis solution according to the manufacturer's instructions. The amount of lysis solution added was equivalent to a final concen-
TABLE 2. Relative cytotoxicity in L2 cells at 2 h postinfection of oyster homogenates in the presence or absence of V. parahaemolyticus FSL-YI-024

<table>
<thead>
<tr>
<th>Dilution</th>
<th>OH alone</th>
<th>OH + APW</th>
<th>Vp alone</th>
<th>OH + Vp</th>
<th>OH + Vp + APW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>4.0 ± 2.6 A</td>
<td>8.15 ± 4.0 A</td>
<td>80.3 ± 5.4 B</td>
<td>81.7 ± 4.8 B</td>
<td>104.7 ± 3.1</td>
</tr>
<tr>
<td>10-fold</td>
<td>3.7 ± 3.8 A</td>
<td>11.9 ± 3.8 A</td>
<td>67.6 ± 7.0 C</td>
<td>65.9 ± 5.8 C</td>
<td>48.8 ± 4.9</td>
</tr>
<tr>
<td>100-fold</td>
<td>-2.2 ± 3.7 A</td>
<td>6.11 ± 2.9 A</td>
<td>17.1 ± 3.7 A</td>
<td>16.8 ± 2.2 A</td>
<td>11.6 ± 3.3</td>
</tr>
</tbody>
</table>

* Values are mean ± standard error of the mean. OH, initial oyster homogenate diluted in PBS; APW, enrichment in alkaline peptone water for 6 h at room temperature; Vp, V. parahaemolyticus FSL-YI-024. Approximately 1.5 × 10^7 and 7.5 × 10^6 CFU of Vp were used in treatments Vp alone and OH+Vp, respectively. The dilutions for OH+Vp were prepared immediately following inoculation. For the OH+Vp+APW treatment, oyster homogenates were inoculated with −7.5 × 10^6 CFU of Vp followed by enrichment in APW for 6 h at room temperature. At the end, the enrichment sample contained ~1.5 × 10^8 CFU/ml. Fifteen microliters of either undiluted (~2.3 × 10^8 CFU) or diluted enrichments was used for the LDH assay. Relative cytotoxicity for treatments OH alone, OH+APW, Vp alone, OH+Vp were analyzed using a two-way ANOVA. Values with different letters are significantly different (P < 0.05).

Results and Discussion

One common strategy for identifying pathogenic bacterial strains is to test for the presence of specific genes or protein products that are correlated with pathogenicity (i.e., virulence markers). Examples of virulence markers include inv, which encodes a mammalian cell invasion factor, and...
ial, which is the invasion-associated locus. The presence of \textit{inv} is associated with pathogenic strains of \textit{Yersinia pseudotuberculosis} (12, 39) and \textit{ial} is highly specific for \textit{Shigella} and enteroinvasive \textit{Escherichia coli} (22). The reliability of virulence markers for differentiation of pathogenic strains is dependent on the universality of the presence of the marker in strains capable of causing disease.

The current standard method for detection of pathogenic \textit{V. parahaemolyticus} strains is based on the presence of \textit{tdh} (encoding the TDH protein) and \textit{trh} (encoding TDH-related hemolysin) (40). Not all pathogenic \textit{V. parahaemolyticus} are positive for \textit{tdh} and \textit{trh}, and the ability of vibrios to exchange genetic material, and genetic markers (1, 33), strongly underscores the need for development of analytical tools that can measure the functional capability of a specific strain to cause disease. We reasoned that a tissue culture approach that allowed measurement of the relative cytopathogenicity of clinical \textit{V. parahaemolyticus} strains and of strains not associated with human illness might provide such a tool. The relative cytotoxicity of \textit{V. parahaemolyticus} isolates was examined by measuring LDH release in several different host cell lines. We also tested the feasibility of using LDH release as a detection method for \textit{V. parahaemolyticus} in seafood samples.

**Cytopathogenicity assays and groupings of isolates.** Because microorganisms interact differently with host tissues from different sources (15, 41), we chose multiple tissue cell lines representing different host cell types, including the HeLa cell line that has been used in previous studies on \textit{V. parahaemolyticus} (28, 35, 47), to measure relative cytotoxicity of the isolates in our \textit{V. parahaemolyticus} collection. Specifically, we measured host cell release of LDH, an indigenous enzyme found in human and animal cells, in the presence of the \textit{V. parahaemolyticus} cultures. The quantity of LDH released is proportional to the degree of damage to the host cell membrane (31) incurred by exposure to \textit{V. parahaemolyticus}.

In \textit{Vibrio cholerae} and \textit{Salmonella}, enterotoxigenicity is associated with production of a factor that causes elongation of CHO cells (7, 36). Therefore, we also conducted the CHO cell elongation assay (i) to determine whether \textit{V. parahaemolyticus} possesses a CHO cell elongation factor and if so (ii) to determine the feasibility of this assay for differentiating between pathogenic and nonpathogenic bacteria.

The isolates used in this study were classified into various categories. Clinical isolates were obtained from patient stool samples, and food isolates (i.e., nonclinical isolates) were obtained from food samples that had not been associated with any clinical cases. Isolates were also classified by serotype into O3:K6 and non-O3:K6 groups. \textit{V. parahaemolyticus} serotype O3:K6 is an emerging pathogen hypothesized to have enhanced virulence relative to non-O3:K6 strains; O3:K6 strains and the closely related clonal groups have caused a number of human disease outbreaks worldwide since 1996 (3, 4, 6, 20, 34). Because TDH has long been regarded as a virulence marker for \textit{V. parahaemolyticus}, we also compared the relative cytotoxicity of isolates with and without \textit{tdh}.

\textit{V. parahaemolyticus} isolates exhibit different cytotoxic capabilities. When grouped by source, food and clinical isolates had different cytotoxic capabilities. For all tissue culture cell lines, the mean cytotoxicity of clinical isolates was greater than that for food isolates (Fig. 1A). Clinical isolates exhibited 54.5, 59.6, 50.0, 36.9, and 15.5% relative cytotoxicity in HeLa, J774.A1, L2, Henle 407, and Caco-2 cell lines, respectively, whereas, food isolates exhibited 43.8, 54.9, 26.2, 22.4, and 4.4% relative cytotoxicity in these respective cell lines. The relative cytotoxicity for these two groups was significantly different \(P < 0.05\).
in the L2, Henle 407, and Caco-2 cell lines. Cytotoxicity of the V. parahaemolyticus O3:K6 and non-O3:K6 isolates also was compared. Because all O3:K6 isolates in our collection were from clinical sources, only clinical non-O3:K6 isolates were compared with clinical O3:K6 isolates. Cytotoxicity differences between these two groups were significant in Henle 407 cells (45.0% with the O3:K6 group versus 23.9% with the non-O3:K6 group), HeLa cells (67.3 versus 31.4%), and J774A.1 cells (68.5 versus 43.5%) (Fig. 1B). The differences in L2 cells (61.0 versus 32.5%) were marginally significant (P < 0.10). Although tdh-positive isolates appeared to have slightly higher cytotoxicity than tdh-negative isolates (Fig. 1C), the differences were significant only for the Caco-2 cell line.

Our findings from the LDH assays support conclusions from previous studies, which suggested that the presence of tdh alone does not fully correlate with cytotoxicity, fluid accumulation, and other virulence measurements (9, 23, 35); therefore, tdh alone is not sufficient as a predictor for V. parahaemolyticus pathogenicity. Our results further suggest that the apparent enhanced virulence of V. parahaemolyticus O3:K6 strains could be associated with the enhanced cytotoxic effects that these cells have on host cells. When classified into groups by source, clinical isolates had higher relative cytotoxicity than did food isolates in three host cell lines. In its current format, however, this LDH assay cannot reliably discriminate between clinical and nonclinical isolates at the strain level because the relative cytotoxicity percentages in these two groups of isolates overlap. Nevertheless, with further development, a tissue culture–based assay may be a useful approach for the detection of pathogenic strains because the outcome of such an assay will be more dependent on the ability of a strain to cause disease than on the presence or absence of one or more specific virulence determinants.

CHO elongation factor is not useful for evaluation of V. parahaemolyticus pathogenicity. Although V. parahaemolyticus isolates clearly differed in their cytotoxic effects on host cells, the organism does not appear to possess a CHO elongation factor under the conditions tested. Nine V. parahaemolyticus isolates (Table 1) were screened for the presence of the CHO elongation factor, but none were positive. Thus, the CHO elongation assay is not useful for characterization of V. parahaemolyticus pathogenicity.

Development of a tissue culture–based assay to detect cytotoxic V. parahaemolyticus. Because clinical isolates generally were more cytotoxic than were nonclinical isolates, we further tested the efficacy of the LDH assay as a detection tool to screen for the presence of V. parahaemolyticus strains with pathogenic potential in food samples. Our goal with these experiments was to assess the ability of our assay to detect the presence of pathogenic V. parahaemolyticus directly from an oyster tissue matrix. The ability to detect potentially dangerous pathogens directly from food samples could significantly reduce the analytical time needed to make decisions related to hazards that may be associated with bacterial contamination, such as determining the safety of oyster beds or the need to issue a product recall. However, food matrices can interfere with analytical outcomes. For example, inhibitory substances present in food can lead to false-negative results with some detection technologies such as those involving PCRs (19). Oyster samples were tested because V. parahaemolyticus infections have been associated frequently with consumption of raw or undercooked bivalve shellfish. We selected L2 cells for assay development because the cytotoxicity difference between clinical and nonclinical isolates was the most pronounced with this cell line, i.e., 6 (46.2%) of 13 clinical isolates but none of the food isolates had >60% relative cytotoxicity. The fast-growing L2 cell line also is easy to maintain. V. parahaemolyticus FSL-Y1-024 was selected for the development of the screening assay because of its relative severe cytotoxic effects in all cell lines (data not shown).

Approximately 5 × 10^5 CFU of FSL-Y1-024 culture was used to artificially inoculate the initial diluted oyster homogenates, and then 15 µl of the mixtures (undiluted or diluted 10- or 100-fold) were used to infect L2 cells either with or without an enrichment step. Results are summarized in Table 2. A low level of cytotoxicity was detected in oyster homogenates and APW (treatments OH alone and OH + APW in Table 2), presumably because of nonspecific LDH release from the oyster samples. This nonspecific LDH release constituted the background level for our assay. When L2 cells were inoculated with 1.5 × 10^7 CFU of V. parahaemolyticus FSL-Y1-024 alone, a strong response (80.3% cytotoxicity) was detected. A reduced response (67.6% cytotoxicity) was detected with 10-fold diluted cultures, i.e., 1.5 × 10^6 CFU. The cytotoxicity of the 100-fold diluted cultures was not significantly different from the background. Components present in oyster homogenates, including endogenous LDH, did not appear to affect detection of V. parahaemolyticus; similar cytotoxicity was detected for FSL-Y1-024 either with or without oyster homogenates (treatments OH + Vp and Vp alone).

Although the addition of an enrichment step to a detection assay increases the overall time required for assay completion, this step can improve assay sensitivity. We demonstrated previously that a 6-h enrichment period was optimum for detecting a larger collection of V. parahaemolyticus with a PCR method (46). Enrichment at an appropriate temperature can allow injured cells to recover and viable-but-nonculturable (VBNC) cells to resuscitate (13, 21, 25). In this regard, an enrichment step may be particularly useful for V. parahaemolyticus because of its ability to enter into the VBNC stage (2, 30). Wong et al. (43) found that VBNC cells exhibited delayed mouse lethality and enteropathogenicity compared with log-phase cells. In the natural environment, V. parahaemolyticus also may be injured or starved. Wong and Chang (42) found that a short incubation was necessary to enable starved V. parahaemolyticus cells to reach the same cytopathogenicity as exhibited by the log-phase cells. To balance the benefits of enrichment with the need for a rapid assay, a 6-h enrichment period was selected for the present study. FSL-Y1-024 numbers increased by 1.26 log CFU following this enrichment. Statistical comparison of the treatment with an
enrichment step (OH+Vp+APW) and the other treatments was not conducted because bacterial cell numbers differed by >0.5 log CFU.

Cytotoxicity measurements following enrichment may not be comparable to those without enrichment. In this study, *V. parahaemolyticus* cells after the 6-h enrichment were likely in late log to early stationary phase in contrast to the late stationary phase cells used in other treatments. There is increasing evidence that virulence of bacterial pathogens such as *Listeria monocytogenes* (15) and *Streptococcus pneumoniae* (17) is influenced by environmental stresses and growth phase. Thus, it is also possible that *V. parahaemolyticus* cytopathogenicity may be influenced by growth phase. Further study will be necessary to assess the effects of growth phase on *V. parahaemolyticus* virulence characteristics.

In its current format, our LDH assay can differentiate clinical *V. parahaemolyticus* strains only at the population level. Future assay optimization also must take into consideration the presence of other microflora in a typical test matrix. Improved sensitivity and specificity will be necessary for application of this approach as a routine detection procedure.

Conventional detection tools designed to establish the presence of one genetic determinant or phenotypic characteristic in a given isolate are likely to be of only limited utility for detecting emerging pathogenic *V. parahaemolyticus* strains. In the present study, we examined the utility of a tissue culture–based assay for development of detection methods for pathogenic *V. parahaemolyticus* strains. As a group, clinical isolates are more cytotoxic than nonclinical isolates. Based on LDH release results, ~10^6 cells of cytotoxic *V. parahaemolyticus* were detected. An optimized enrichment step might further enhance the sensitivity of this assay. Further insight into factors contributing to *V. parahaemolyticus* pathogenesis will enable development of new detection methods with increased sensitivity and discriminatory capabilities.

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