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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 cytopathogenicity. 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 $\sim 1.2 \times 10^6$ 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	*tdh* PCR[a]
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-Y1-005[b]	5C-1C	O1	Food (oyster)	Washington	1988	−
FSL-Y1-006	M350A	O5	Food (oyster)	Washington	1994	−
FSL-Y1-010[b]	T-3979	O5:K15	Clinical	Japan	Unknown	+
FSL-Y1-011[b]	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-Y1-014[b]	AN-5034	O4:K68[c]	Clinical	Bangladesh	1996	+
FSL-Y1-015	AN-16000	O1:KUT[cd]	Clinical	Bangladesh	1998	+
FSL-Y1-016	TX-2103	O3:K6	Clinical	Texas	1998	+
FSL-Y1-017[b]	NY-3064	O3:K6	Clinical	New York	1998	+
FSL-Y1-021[b]	U-5474	Old O3:K6[e]	Clinical	Bangladesh	1980	+
FSL-Y1-023[b]	VP86	O3:K6	Clinical	Calcutta	1996	+
FSL-Y1-024[b]	VP199	O3:K6	Clinical	Calcutta	1997	+
FSL-Y1-025	VP208	O3:K6	Clinical	Calcutta	1997	+
FSL-Y1-026[b]	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:KUT[d]	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 umpublished data.
[b] Isolates tested for CHO elongation activity.
[c] Strains deemed genetically similar to O3:K6 strains by arbitrarily primed PCR, ribotypes, and pulsed-field gel electrophoresis pattern analyses *(5, 26)*.
[d] UT, untypeable.
[e] 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 were subcultured on TSAS at least once before each experiment.

Tissue cell lines. *V. parahaemolyticus* cytotoxicity was determined in human epithelial HeLa cells (ATCC CCL-2), mouse macrophage-like J774A.1 cells (ATCC TIB-67), human epithelial Henle 407 cells (ATCC CCL-6), rat epithelial L2 cells (ATCC CCL-149), and the human colorectal epithelial cells Caco-2 (ATCC HTB-37). The HeLa, J774A.1, Henle 407, and L2 cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Invitrogen, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS; Gibco). Caco-2 cells were maintained in minimal essential medium (Gibco) supplemented with 20% FBS. The CHO elongation factor assays were conducted using Chinese hamster ovary cells CHO-1C6 (ATCC CRL-1793) that were maintained in Hams F12K medium (Gibco) with 10% FBS. All tissue cells were incubated at 37°C with 5% CO_2.

Cytotoxicity assays. A tissue culture–based assay was used to measure host cell cytotoxicity induced in the presence of stationary-phase *V. parahaemolyticus* cells. A 500-μl aliquot of *V. parahaemolyticus* cells (8×10^8 to 2×10^9 CFU/ml, as determined by standard plate count in each experiment) that had been grown overnight in TSBS with shaking (250 rpm) at 37°C was centrifuged at 17,900 × *g* for 3 min, and the pellet was resuspended in 500 μl of phosphate-buffered saline (PBS). The resuspension was diluted 10-fold in PBS, and a 15-μl aliquot (i.e., to yield 1.5 μl of the undiluted suspension) was used to infect confluent monolayers of tissue cells grown in 100 μl of the appropriate medium in 96-well microtiter plates (Fisher Scientific International, Inc., Hampton, NH). At 2 h postinfection, cytotoxicity was assessed by measurement of released host cell LDH using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wis.). LDH concentration was measured by reading absorbance at 490 nm using the Fusion Universal microplate analyzer (Packard Instrument Co., Meriden, Conn.). Relative cytotoxicity calculations were based on the following formula: cytotoxicity (%) = $100 \times (A_{sample} - A_{spontaneous})/(A_{total} - A_{spontaneous})$, where A_{sample} is the absorbance of infected tissue cells and $A_{spontaneous}$ is the absorbance of uninfected tissue cells. To determine A_{total}, the maximal release of LDH, tissue cells were lysed by adding 15 μl 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-Y1-024*

Dilution	Relative cytotoxicity (%)[a]				
	OH alone	OH+APW	Vp alone	OH+Vp	OH+Vp+APW
Undiluted	4.0 ± 2.6 A	8.15 ± 4.0 A	80.3 ± 5.4 B	81.7 ± 4.8 B	104.7 ± 3.1
10-fold	3.7 ± 3.8 A	11.9 ± 3.8 A	67.6 ± 7.0 C	65.9 ± 5.8 C	48.8 ± 4.9
100-fold	−2.2 ± 3.7 A	6.11 ± 2.9 A	17.1 ± 3.7 A	16.8 ± 2.2 A	11.6 ± 3.3

[a] 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-Y1-024. 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 (~2.3×10^6 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 ($P < 0.05$).

tration of 1% Triton X-100. With each cell line, each isolate was tested in multiple independent experiments resulting in at least 18 replicates.

CHO elongation factor assays. Culture supernatants of *V. parahaemolyticus* strains were tested for CHO elongation activity *(18, 27).* Approximately 10^3 CHO cells were grown for 24 h in 100 μl of Hams F12K medium with 10% FBS in 96-well microtiter plates. Cells were infected with 15 μl of each *V. parahaemolyticus* overnight culture supernatant or with purified TDH at concentrations ranging from 1 to 150 μg/ml (Sigma-Aldrich, St. Louis, Mo.). At specific postinfection time points, the number of elongated CHO cells in the presence of each *V. parahaemolyticus* culture supernatant was scored relative to the number of nontreated cells (negative control) or of cells treated with 5 ng/ml of cholera toxin (Sigma) (positive control). Positive and negative controls were run concomitantly in all experiments. The number of elongated cells in the presence of cholera toxin was defined as 100% in each assay. At least three independent experiments were conducted for each *V. parahaemolyticus* isolate. Isolates that yielded >50% elongated CHO cells were considered positive.

Cytotoxicity of oyster homogenates. To determine whether the presence of oyster tissue would influence *V. parahaemolyticus*–induced tissue culture cytotoxicity measurements, the cytotoxicity assays on L2 cells were conducted with a mixture of 1:10 diluted oyster homogenates and a highly cytotoxic *V. parahaemolyticus* strain. Preparation of oyster samples was as described previously *(46).* Canned oysters purchased from a local grocery store were blended and diluted with PBS to make 1:10 diluted oyster homogenates. These homogenates were treated with UV radiation to kill endogenous microflora and stored at −20°C until use and are referred to hereinafter as the initial diluted oyster homogenates. The highly cytotoxic *V. parahaemolyticus* strain was FSL-Y1-024. To prepare the bacterial culture, a 1-ml aliquot of FSL-Y1-024 that had been grown overnight (at 37°C and 250 rpm) was washed and resuspended in 1 ml of PBS.

We first tested the effect of oyster homogenate alone on L2 cell cytotoxicity. A 1-ml aliquot of the initial diluted oyster homogenate was centrifuged at 17,900 × *g* for 4 min, and the pellet was resuspended in 1 ml of PBS. This suspension was further diluted 10- and 100-fold with PBS, and 15 μl of the undiluted and each diluted suspension was used to infect L2 cells. The effect of bacterial culture alone on L2 cells cytotoxicity was tested as described above, except that 15 μl of *V. parahaemolyticus* FSL-Y1-024, either undiluted (containing approximately 1.5×10^7 CFU) or diluted (10- or 100-fold in PBS) was used to infect L2 cells.

To prepare an oyster homogenate and bacterial culture mixture, 500 μl of the initial diluted oyster homogenate and 500 μl of a washed overnight culture of FSL-Y1-024 were mixed and then centrifuged at 17,900 × *g* for 4 min. This undiluted mixture contained approximately 5.0×10^8 CFU/ml of FSL-Y1-024. The pellet was resuspended with 1 ml of PBS, and 10- and 100-fold dilutions were prepared. For each of the undiluted and diluted mixtures, 15 μl was used to infect L2 cells as described above. The undiluted infected mixture contained approximately 7.5×10^6 CFU of FSL-Y1-024.

To determine the effect of an enrichment step on assay efficacy, a mixture of 500 μl of initial diluted oyster homogenate and 500 μl of bacterial culture were incubated statically with 30 ml of alkaline peptone water (APW) at 37°C for 6 h. Following the enrichment incubation period, the suspension was mixed thoroughly, and 1 ml was transferred to a 1.5-ml eppendorf tube. The suspension was washed once and then resuspended with 1 ml of PBS, and 15 μl of the undiluted and diluted (10- and 100-fold) enrichment samples was used to infect L2 cells. The undiluted enrichment sample was evaluated for FSL-Y1-024 concentration, which was approximately 1.5×10^8 CFU/ml. Therefore, the undiluted infected mixture contained approximately 2.3×10^6 CFU. As a negative control, the initial diluted oyster homogenates and APW mixture was tested as described above but without addition of a bacterial culture. For all treatments, each sample was run at least in quadruplicate in three independent experiments.

Statistical analyses. The differences in cytotoxicity between food and clinical isolates, *tdh*-positive and *tdh*-negative isolates, and O3:K6 and non-O3:K6 *V. parahaemolyticus* strains were analyzed using the two-sample *t* test and the Mann-Whitney test for normally and nonnormally distributed data, respectively (Minitab Inc., State College, Pa.). Differences in cytotoxicity among treatments in Table 2 were analyzed by two-way analysis of variance (ANOVA; Minitab Inc.). Differences were considered significant at $P < 0.05$.

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 *inv* is associated with pathogenic strains of *Yersinia pseudotuberculosis (12, 39)* and *ial* is highly specific for *Shigella* and enteroinvasive *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 *V. parahaemolyticus* strains is based on the presence of *tdh* (encoding the TDH protein) and *trh* (encoding TDH-related hemolysin) *(40).* Not all pathogenic *V. parahaemolyticus* are positive for *tdh* and *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 *V. parahaemolyticus* strains and of strains not associated with human illness might provide such a tool. The relative cytotoxicity of *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 *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 *V. parahaemolyticus (28, 35, 47),* to measure relative cytotoxicity of the isolates in our *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 *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 *V. parahaemolyticus.*

In *Vibrio cholerae* and *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 *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. *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 *V. parahaemolyticus (29, 32),* we also compared the relative cytotoxicity of isolates with and without *tdh.*

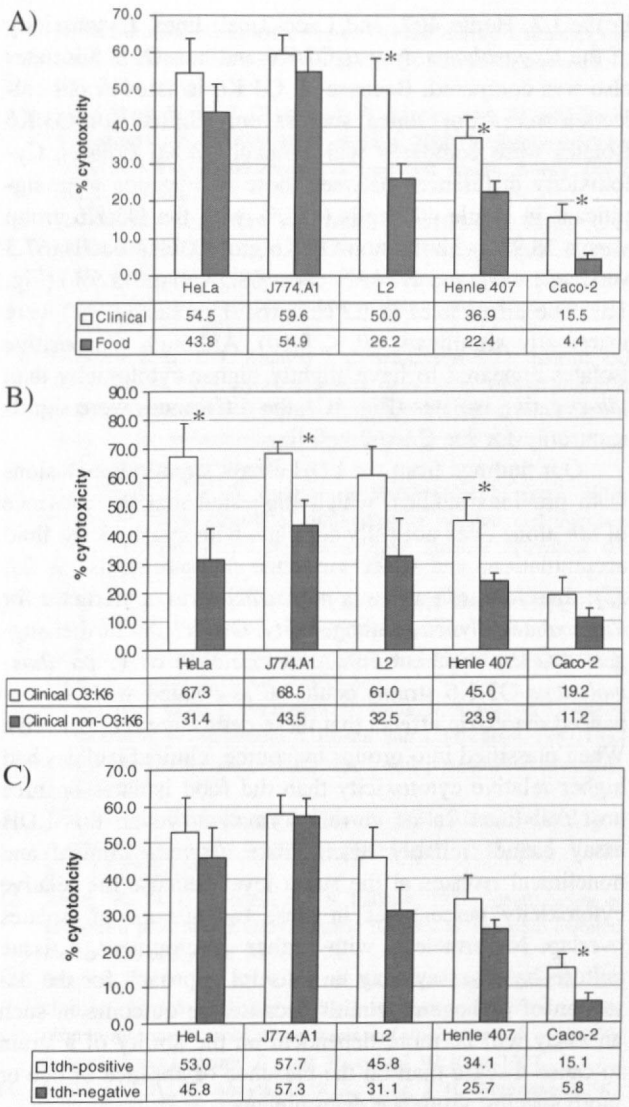

FIGURE 1. *Cytotoxicity (%, relative to complete chemical cell lysis) in different cell lines at 2 h postinfection with* V. parahaemolyticus *isolates. Cytotoxicity was measured by host LDH release. Error bars represent the standard error of the mean. Mean cytotoxicity percentages are noted in tables below each figure. For statistical analyses, isolates were grouped: (A) food and clinical isolates; (B) clinical non-O3:K6 versus O3:K6 isolates; and (C)* tdh^+ *versus* tdh^- *isolates. An asterisk indicates that the cytotoxicity difference between each group for a given cell line is significantly different* ($P < 0.05$).

***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 5×10^8 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, $\sim 10^6$ 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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REFERENCES

1. Baba, K., H. Shirai, A. Terai, K. Kumagai, Y. Takeda, and M. Nishibuchi. 1991. Similarity of the *tdh* gene–bearing plasmids of *Vibrio cholerae* non-O1 and *Vibrio parahaemolyticus*. *Microb. Pathog.* 10: 61–70.
2. Bates, T. C., and J. D. Oliver. 2004. The viable but nonculturable state of Kanagawa positive and negative strains of *Vibrio parahaemolyticus*. *J. Microbiol.* 442:74–79.
3. Chiou, C. S., S. Y. Hsu, S. I. Chiu, T. K. Wang, and C. S. Chao. 2000. *Vibrio parahaemolyticus* serovar O3:K6 as cause of unusually high incidence of food-borne disease outbreaks in Taiwan from 1996 to 1999. *J. Clin. Microbiol.* 38:4621–4625.
4. Chowdhury, A., M. Ishibashi, V. D. Thiem, D. T. Tuyet, T. V. Tung, B. T. Chien, L. von Seidlein, D. G. Canh, J. Clemens, D. D. Trach, and M. Nishibuchi. 2004. Emergence and serovar transition of *Vibrio parahaemolyticus* pandemic strains isolated during a diarrhea outbreak in Vietnam between 1997 and 1999. *Microbiol. Immunol.* 48: 319–327.
5. Chowdhury, N. R., S. Chakraborty, T. Ramamurthy, M. Nishibuchi, S. Yamasaki, Y. Takeda, and G. B. Nair. 2000. Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. *Emerg. Infect. Dis.* 6:631–636.
6. Daniels, N. A., B. Ray, A. Easton, N. Marano, E. Kahn, A. L. McShan II, L. Del Rosario, T. Baldwin, M. A. Kingsley, N. D. Puhr, J. G. Wells, and F. J. Angulo. 2000. Emergence of a new *Vibrio parahaemolyticus* serotype in raw oysters: a prevention quandary. *JAMA* 284:1541–1545.
7. Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman. 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. *Infect. Immun.* 10:320–327.
8. Hackney, C. R., E. G. Kleeman, B. Ray, and M. L. Speck. 1980. Adherence as a method of differentiating virulent and avirulent strains of *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.* 40: 652–658.
9. Hoashi, K., K. Ogata, H. Taniguchi, H. Yamashita, K. Tsuji, Y. Mizuguchi, and N. Ohtomo. 1990. Pathogenesis of *Vibrio parahaemolyticus*: intraperitoneal and orogastric challenge experiments in mice. *Microbiol. Immunol.* 34:355–366.
10. Hondo, S., I. Goto, I. Minematsu, N. Ikeda, N. Asano, M. Ishibashi, Y. Kinoshita, N. Nishibuchi, T. Honda, and T. Miwatani. 1987. Gastroenteritis due to Kanagawa negative *Vibrio parahaemolyticus*. *Lancet* i:331–332.
11. Iijima, Y., H. Yamada, and S. Shinoda. 1981. Adherence of *Vibrio parahaemolyticus* and its relation to pathogenicity. *Can. J. Microbiol.* 27:1252–1259.
12. Isberg, R., D. L. Voorhis, and S. Falkow. 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell* 50:769–778.
13. Johnston, M. D., and M. H. Brown. 2002. An investigation into the changed physiological state of *Vibrio* bacteria as a survival mechanism in response to cold temperatures and studies on their sensitivity to heating and freezing. *J. Appl. Microbiol.* 92:1066–1077.
14. Kaysner, C. A., C. Abeyta, Jr., P. A. Trost, J. H. Wetherington, K. C. Jinneman, W. E. Hill, and M. M. Wekell. 1994. Urea hydrolysis can predict the potential pathogenicity of *Vibrio parahaemolyticus* strains isolated in the Pacific Northwest. *Appl. Environ. Microbiol.* 60:3020–3022.
15. Kim, H., H. Marquis, and K. J. Boor. 2005. σ^B contributes to *Listeria monocytogenes* invasion by controlling expression of *inlA* and *inlB*. *Microbiology* 151: 3215–3222.
16. Kishishita, M., N. Matsuoka, K. Kumagai, S. Yamasaki, Y. Takeda, and M. Nishibuchi. 1992. Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene of *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.* 58:2449–2457.
17. Ko, K. S., S. Park, W. S. Oh, J. Y. Suh, T. Oh, S. Ahn, J. Chun, and J. H. Song. 2006. Comparative analysis of growth-phase dependent gene expression in virulent and avirulent *Streptococcus pneumoniae* using a high-density DNA microarray. *Mol. Cells* 21:82–88.
18. Kothary, M., E. F. Claverie, M. D. Miliotis, J. M. Madden, and S. H. Richardson. 1995. Purification and characterization of a Chinese hamster ovary cell elongation factor of *Vibrio hollisae*. *Infect. Immun.* 63:2418–2423.
19. Lantz, P. G., W. Abu al-Soud, R. Knutsson, B. Hahn-Hagerdal, and P. Radstrom. 2000. Biotechnical use of polymerase chain reaction for microbiological analysis of biological samples. *Biotechnol. Annu. Rev.* 5:87–130.
20. Laohaprertthisan, V., A. Chowdhury, U. Kongmuang, S. Kalnauwakul, M. Ishibashi, C. Matsumoto, and M. Nishibuchi. 2003. Prevalence and serodiversity of the pandemic clone among the clinical strains of *Vibrio parahaemolyticus* isolated in southern Thailand. *Epidemiol. Infect.* 130:395–406.
21. Liao, C. H., and W. F. Fett. 2005. Resuscitation of acid-injured *Sal-*

monella in enrichment broth, in apple juice and on the surfaces of fresh-cut cucumber and apple. *Lett. Appl. Microbiol.* 41:487–492.
22. Lindqvist, R. 1999. Detection of *Shigella* spp. in food with a nested PCR method—sensitivity and performance compared with a conventional culture method. *J. Appl. Microbiol.* 86:971–978.
23. Lynch, T., S. Livingstone, E. Buenaventura, E. Lutter, J. Fedwick, A. G. Buret, D. Graham, and R. DeVinney. 2005. *Vibrio parahaemolyticus* disruption of epithelial cell tight junctions occurs independently of toxin production. *Infect. Immun.* 73:1275–1283.
24. Makino, K., K. Oshima, K. Kurokawa, K. Yokoyama, T. Uda, K. Tagomori, Y. Iijima, M. Najima, M. Nakano, A. Yamashita, Y. Kubota, S. Kimura, T. Yasunaga, T. Honda, H. Shinagawa, M. Hattori, and T. Iida. 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *Lancet* 361: 743–749.
25. Ma-Lin, C. F. A., and L. R. Beuchat. 1980. Recovery of chill-stressed *Vibrio parahaemolyticus* from oysters with enrichment broths supplemented with magnesium and iron salts. *Appl. Environ. Microbiol.* 39:179–185.
26. Matsumoto, C., J. Okuda, M. Ishibashi, M. Iwanaga, P. Garg, T. Rammamurthy, H. C. Wong, A. Depaola, Y. B. Kim, M. J. Albert, and M. Nishibuchi. 2000. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *tox*RS sequence analyses. *J. Clin. Microbiol.* 38:578–585.
27. McCardell, B. A., M. H. Kothary, R. H. Hall, and V. Sathyamoorthy. 2000. Identification of a CHO cell-elongating factor produced by *Vibrio cholerae* O1. *Microb. Pathog.* 29:1–8.
28. Merrell, B. R., R. I. Walker, and S. W. Joseph. 1984. In vitro and in vivo pathologic effects of *Vibrio parahaemolyticus* on human epithelial cells. *Can. J. Microbiol.* 30:381–388.
29. Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai. 1969. In vitro hemolytic characteristics of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. *J. Bacteriol.* 100:1147–1149.
30. Mizunoe, Y., S. N. Wai, T. Ishikawa, A. Takade, and S. Yoshida. 2000. Resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under starvation. *FEMS Microbiol. Lett.* 186:115–120.
31. Nachlas, M. M., S. I. Margulies, J. D. Goldberg, and A. M. Seligman. 1960. The determination of lactic dehydrogenase with a tetrazolium salt. *Anal. Biochem.* 10:317–326.
32. Nishibuchi, M., A. Fasano, R. G. Russell, and J. B. Kaper. 1992. Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct hemolysin. *Infect. Immun.* 60: 3539–3545.
33. Nishibuchi, M., and J. B. Kaper. 1990. Duplication and variation of the thermostable direct haemolysin (*tdh*) gene in *Vibrio parahaemolyticus*. *Mol. Microbiol.* 4:87–99.
34. Okuda, J., M. Ishibashi, E. Hayakawa, T. Nishino, Y. Takeda, A. K. Mukhopadhyay, S. Garg, S. K. Bhattacharya, G. B. Nair, and M. Nishibuchi. 1997. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. *J. Clin. Microbiol.* 35:3150–3155.
35. Park, K.-S., T. Ono, M. Rokuda, M.-H. Jang, T. Iida, and T. Honda. 2004. Cytotoxicity and enterotoxicity of the thermostable direct hemolysin-deletion mutants of *Vibrio parahaemolyticus*. *Microbiol. Immunol.* 48:313–318.
36. Rumeu, M. T., M. A. Suarez, S. Morales, and R. Rotger. 1997. Enterotoxin and cytotoxin production by *Salmonella* Enteritidis strains isolated from gastroenteritis outbreaks. *J. Appl. Microbiol.* 82:19–31.
37. Shirai, H., H. Ito, T. Hirayama, Y. Nakamoto, N. Nakabayashi, K. Kumagai, Y. Takeda, and M. Nishibuchi. 1990. Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis. *Infect. Immun.* 58:3568–3573.
38. Sochard, M. R., and R. R. Colwell. 1977. Toxin isolation from a Kanagawa-phenomenon negative strain of *Vibrio parahaemolyticus*. *Microbiol. Immunol.* 21:243–254.
39. U.S. Food and Drug Administration. 2001. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. *In* Bacteriological analytical manual online. Available at: http://www.cfsan.fda.gov/~ebam/bam-8.html. Posted January 2001.
40. U.S. Food and Drug Administration. 2004. Bacteriological analytical manual online. Available at: http://www.cfsan.fda.gov/~ebam/bam-9.html. Posted May 2004.
41. Venkateswaran, K., H. Horiuchi, H. Nakano, H. Matsuda, and H. Hashimoto. 1991. *Vibrio* virulence factors and the quantitative analysis of cytotoxicity elaborated by environmental isolates. *Cytobios* 66:143–151.
42. Wong, H.-C., and C.-N. Chang. 2005. Hydrophobicity, cell adherence, cytotoxicity, and enterotoxigenicity of starved *Vibrio parahaemolyticus*. *J. Food Prot.* 68:154–156.
43. Wong, H.-C., C.-T. Shen, C.-N. Chang, Y.-S. Lee, and J. Oliver. 2004. Biochemical and virulence characterization of viable but nonculturable cells of *Vibrio parahaemolyticus*. *J. Food Prot.* 67:2430–2435.
44. Yamamoto, S., N. Okujo, S. Miyoshi, S. Shinoda, and S. Narimatsu. 1999. Siderophore production of *Vibrio parahaemolyticus* strains from different sources. *Microbiol. Immunol.* 43:909–912.
45. Yeung, P. S. M., and K. J. Boor. 2004. Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. *Foodborne Pathog. Dis.* 1:74–88.
46. Yeung, P. S. M., A. DePaola, C. A. Kaysner, and K. J. Boor. 2003. A PCR assay for specific detection of the pandemic *Vibrio parahaemolyticus* O3:K6 clone from shellfish. *J. Food Sci.* 68:1459–1466.
47. Yeung, P. S. M., M. C. Hayes, A. DePaola, C. A. Kaysner, L. Kornstein, and K. J. Boor. 2002. Comparative phenotypic, molecular and virulence characterization of *Vibrio parahemolyticus* O3:K6 isolates. *Appl. Environ. Microbiol.* 68: 2901–2909.