Survey of clinical and environmental *Vibrio parahaemolyticus* isolates for putative virulence factors and cytotoxicity of extracellular products to a CHO-K1 cell line

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Abstract

The halophilic bacterium *Vibrio parahaemolyticus* is widely distributed as a natural inhabitant of marine and estuarine environments throughout the world. Many strains are avirulent in humans, but virulent strains are the causative agent of gastroenteritis acquired through consumption of contaminated raw or undercooked seafood. Therefore, it is likely that most *V. parahaemolyticus* isolates from the marine environment are harmless to humans. Virulent isolates are difficult to differentiate from the majority of avirulent environmental isolates. A total of 55 *V. parahaemolyticus* isolates (22 clinical, 31 environmental and 2 reference strains) were examined for the carriage of plasmids, whole cell protein profiles; their haemolytic, lipase, phospholipase, protease and urease activities; and the cytotoxicity of their extracellular products (ECPs). Analysis of variance for *V. parahaemolyticus* isolates showed that Kanagawa haemolysis, phospholipase, protease and urease activities were significantly related to cytotoxicity as assayed by measuring relative extracellular lactate dehydrogenase as an indicator of damage of CHO-K1 cells. The difference between the mean values of cytotoxicity of ECPs of clinical and environmental isolates was significant at a $p$ value of $< 0.05$.

Key words: Cytotoxicity; enzyme activity; *V. parahaemolyticus*; virulence factors

1-Introduction

*Vibrio parahaemolyticus* is an important enteric pathogen associated with fish and shellfish consumption. Since the first discovery of *V. parahaemolyticus* during a gastroenteritis outbreak in Japan in 1951, it has been found to be widely distributed (1), and is found in coastal waters in temperate zones and can be readily isolated from seafood (2). Pathogenic *V. parahaemolyticus* strains that cause gastroenteritis due to consumption of seafood that is contaminated or infected by this organism has gained significant worldwide attention due to the increasing trend of human incidences of food poisoning.

In Northern Europe, potentially pathogenic *V. parahaemolyticus* are still relatively rare (3) but with the advent of global warming this may change, but quick identification and detection methods for this bacterium are lacking (4). Moreover, only certain strains are able to cause disease and the majority of environmental isolates are avirulent. Although several possible
virulence factors have been described, the overall mechanism of pathogenesis by *V. parahaemolyticus* remains poorly understood (5).

Most *V. parahaemolyticus* clinical isolates exhibit the Kanagawa phenomenon (KP) beta-type haemolysis on Wagatsuma agar due to production of thermostable direct haemolysin (TDH) (6). Some isolates also carry TDH-related haemolysin (TRH). Genes for these haemolysins (*tdh* and *trh*) share around 70\% nucleotide sequence identity (7). However, there is limited knowledge on how TDH and TRH contribute to disease.

The KP is currently used as a clinical indicator for the virulence of *V. parahaemolyticus* and has been recognized as a marker for discriminating pathogenic from non-pathogenic strains (2). Miyamoto, et al., (8) have reported that beta-type haemolysis on Wagatsuma agar medium was associated with most clinical strains but with very few environmental strains. However, Izutsu et al., (6) reported that human pathogenic *V. parahaemolyticus* isolates include both KP-positive and KP-negative strains. Moreover, many KP-negative strains produce TRH, (9) concluded that TDH alone may not be an absolute indicator of pathogenicity. Furthermore, a TDH-TRH strain caused an outbreak of gastroenteritis in the Maldives Islands in 1985 (10, 11).

Thus, strains carrying *tdh*, or *trh* or both genes are strongly associated with gastroenteritis (12). However, Xu et al., (13) found that virulent *V. parahaemolyticus* lacking *tdh* and *trh* remained pathogenic, suggesting that other virulence factors exist. Proteases are also considered possible virulence factors of pathogenic *V. parahaemolyticus* (8, 14). More recently type III secretion systems (T3SS2) have been investigated as a virulence factor (15).

Although most reported studies on the cytotoxicity of *V. parahaemolyticus* are related to TDH or TRH (16, 17). Lynch et al. (18) have reported that this bacterium has cytotoxic activity even in the absence of TDH and TRH. TDH possesses several biological activities including cytotoxicity, haemolysis, enterotoxicity, and cardiotoxicity (2, 17). TDH, is a membrane-active toxin which causes cell death (16). TDH not only causes haemolysis but also induces morphological changes in other cells eg. loss of the number of microvilli on the cell surface, followed by loss of cytoplasmic substances resulting in cell death (19). Cytotoxicity of TDH may play a role in destroying intestinal epithelial cells, which may lead to a bloody mucous stool (2). Elston and Leibovitz, (20) reported that there is a relationship between pathogenic *Vibrio* spp. isolated from oysters and their ability to produce extracellular products (ECPs). A large number of environmental *Vibrio* spp. produce extracellular toxic factors (21). Zinc metalloprotease has been produced by pathogenic vibrios but in *V. parahaemolyticus* has not been yet determined. Serine protease (VPP1) has been purified from ECPs *V. parahaemolyticus* and its molecular weight found to be 50 kDa (22). Moreover, Lee et al., (23) have purified serine protease (protease A) from ECPs *V. parahaemolyticus* and its molecular weight found to be 43 kDa, it is suggested to play a role in pathogenicity of *V. parahaemolyticus*.

This study aimed to compare clinical and environmental isolates of *Vibrio parahaemolyticus* for activities of a variety of enzymes that may contribute to pathogenicity, and tested extracellular products (ECPs) for cytotoxicity, and tested for the presence of certain genetic markers by PCR, in order to differentiate clinical and environmental (mainly avirulent)
isolates of this bacterium. Strains were also screened for possession of plasmids and protein profiles, using sodium dodecyl sulphate - poly acrylamide gel electrophoresis (SDS-PAGE).

2-Materials and methods

2-1-Bacterial cultures and preparation of ECPs. Fifty-five strains of V. parahaemolyticus from a diverse range of geographical locations were used in this study (Table 1). The cultures were grown on tryptone soya agar or broth (TSA, TSB; Oxoid, Basingstoke, UK) supplemented with 3% (w/v) sodium chloride at 37 °C. Stock cultures were stored as slopes at room temperature (21°C). Tubes containing 10 ml of TSB were inoculated with bacteria from single colonies from 24 h cultures on TSA and incubated at 37 °C for 48 h with shaking. The cultures were centrifuged at 2,800 x g for 15 min at 4°C, and the supernatants obtained after transferring to a fresh tube and re-centrifuging at 2,800 x g for a further 15 min. Minicon B15 Clinical Sample Concentrators (Millipore, USA) were used to concentrate the supernatants according to the manufacturer’s instructions.

2-2-PCR detection of tdh and trh gene sequences. Genomic DNA from bacteria was extracted by using a genomic DNA extraction kit (Qiagen, Ltd., UK) according to the manufacturer’s instructions. PCR assay using primers (5'-CCACTACCACCTCATATGC-3' and 5'-GGTACTAAATGGCTGACATC-3') for tdh, and (5'-GGCTCAAAATGGTTAGCG-3' and 5' CATTTCGCTCATATGC-3') for trh (24).

The PCR was performed using 20 ng of bacterial DNA in a 50 μl reaction volume containing 50 μl of each primer, each deoxy nucleoside triphosphate at a concentration of 0.2 mM, 0.5 unit (U) of Taq DNA polymerase, 1x reaction buffer containing 1.5 mM MgCl₂ (Roche Applied Science, UK), and the reaction volume was made up to 50 μl using PCR grade water. PCR conditions started with an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 70°C for 2 min, and a final extension at 70 °C for 5 min thermal cycler MWG (UK). The amplified PCR products were electrophoresed in 1.5 % agarose gels submerged in 1x TBE buffer (pH 8); with 100 bp DNA ladder (Invitrogen, UK). Gel electrophoresis was performed at 90 V for 3 h by using Pharmacia tanks (Biotech, Sweden). The bands stained with ethidium bromide (EtBr, 0.5 μg/ml) were visualized with UV light (309 nm) using a trans-illuminator and gels were recorded as digital TIFF images using a gel documentation system (UVI-Tech). A negative control without DNA was included in each run to monitor for contamination.

2-3-Screening for plasmids. Isolates of V. parahaemolyticus were screened for plasmid DNA according to the procedures of (25) and (26). The plasmids were visualized on a UV transilluminator (Photodyne), and the gels were photographed by UV light (309 nm) with a Gel Documentation system (Uvi-Tech, UK).

2-4-SDS-PAGE of whole-cell protein profiles. Cultures grown overnight in TSB were pelleted by centrifugation at 1,200 x g for 10 min. Bacterial pellets were resuspended in 50 μl of 2x sample buffer (2 % sodium dodecyl sulphate, 1.25 % 2-mercaptoethanol, 0.1 % bromophenol blue, 2.5 % glycerol in 0.5 mM Tris-C1 at pH 6.8), boiled for 5 min, and centrifuged for 5 min. Approximately 16 μl of the protein sample was used for SDS-PAGE containing 5% stacking and 12.5% of separating gels with a discontinuous buffer system (27), using a vertical slab unit at 125 V constant voltage until the tracking dye left the bottom of the gel. Gels were stained with Coomassie brilliant blue R-250 (Fisher Scientific Ltd., UK). A
Colour Burst™ Electrophoresis Marker (Sigma, UK) was used to estimate the molecular weight of protein bands.

2-5-Assay of KP haemolytic activity. KP haemolysis was examined as previously described (28, 29). Briefly, aliquots (10µl) of overnight cultures were streaked on to Wagatsuma agar medium containing 5% rabbit erythrocytes and grown at 37 °C for 48 h. The isolates exhibiting clear beta-haemolytic zones were designed as positive for the Kanagawa phenomenon. For confirmation, the test was performed in duplicate.

2-6-Assays of enzyme activities. Lipase activity was determined by inoculating the isolates on a lipase assay medium containing (g/l): peptone, 10; NaCl, 10; CaCl₂, H₂O, 0.1; agar, 20, and 1% Tween 80 and incubated at 37°C for 48 h. A white precipitation around the colonies indicated lipase activity. Protease activity was measured on a skim milk agar medium containing (g/l): skim milk, 15; NaCl, 10; agar, 15 and incubated at 37 °C for 48h. A positive response was recorded as the presence of clear zones around the bacterial colonies. Phospholipase activity was measured by inoculating the isolates on a lipase assay medium containing (g/l): - peptone, 10; NaCl, 30; CaCl₂H₂O, 0.1; agar, 15, containing 2.5% egg yolk and incubated at 37 °C for 48 h. A white precipitation around the colonies indicated phospholipase activity. This method was a modification from (30). Urease activity was observed on urea agar plates, (g/l):- agar, 15; NaCl, 30; Na₂HPO₄, 1.2; peptone, 1; glucose, 1; KH₂PO₄, 0.8; phenol red, 0.012. To which 50 ml of sterile 40% urea solution was added after cooling (31). Release of ammonia due to urease activity was detected by a change of pH indicator colour from yellow to pink.

2-7-Cell culture and cytotoxicity assay. Chinese hamster ovary (CHO-K1) cells were cultured in 25 ml flasks in Ham nutrient mixture F-12 supplemented with 10% (v/v) foetal bovine serum (FBS) at 37°C for 72 h. Cytotoxicity of the concentrated ECPs of all V. parahaemolyticus isolates was determined in the CHO-K1 cell cultures by release of lactate dehydrogenase (LDH) using the CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega, UK) based on the manufacturer’s instructions. After confluent growth of the cells in 25 ml flasks, they were washed twice with phosphate-buffered saline (PBS) PBS and treated with 0.25% (w/v) trypsin. Approximately 5x10⁴ trypsinized cells in 100 µl of the medium were dispensed in each well of 96-well microtiter plates (Fisher Scientific International, Inc., UK), and 15 µl of concentrated ECPs was added to the each well of 96-well plates. After 4 h, the supernatants were collected, after centrifugation at 500 x g for 5 min. LDH concentration was measured by reading absorbance at 490 nm using the Fusion Universal microplate analyzer (Optimax, Molecular Devices UK Ltd.) Cytotoxicity was calculated using the equation:

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\frac{([A \text{ sample} - A \text{ spontaneous}])}{([A \text{ total} - A \text{ spontaneous}])} \times 100
\]

Where A sample is the optical density at 490nm (OD490) of the treated cells, A spontaneous is the OD490 reading of untreated cells, and A total is the OD490 reading of treated cells with the lysis buffer (1% Triton X-100) for maximal LDH release.

2-8-Statistical analysis. Statistical analysis was performed using SPSS 18.0 software (SPSS; Plymouth University, UK). A \( p < 0.05 \) was considered statistically significant. The differences in cytotoxicity between clinical and environmental isolates were analysed using \( t \) test.
3-Results

3-1-Plasmid and SDS-PAGE profiles. Plasmids (of varying size) were detected in 8 of 23 (34 %) clinical isolates and 10 of 32 (31 %) environmental isolates (Table 1). SDS-PAGE of whole cell soluble proteins failed to differentiate clinical and environmental V. parahaemolyticus isolates; although there were slight deviations in the number of bands generated, the profiles for all isolates tested generally remained the same (example profiles are shown in Fig 1 for a limit number of isolates.

3-2-Enzyme activities and presence of tdh and trh. Table 1 also shows the results of tests for enzyme activities and the presence of tdh and trh sequences as determined by PCR.

Overall, 83% of clinical isolates and 16% of environmental isolates exhibited a clear beta-haemolysis on Wagatsuma agar medium and can be considered as KP-positive. All the clinical isolates and 47% of the environmental isolates tested were able to hydrolyse urea, whilst 26% of clinical isolates and 16% environmental isolates were positive for the lipase assay. Protease activity was demonstrated by 83% of clinical isolates and 44% of environmental isolates. Phospholipase activity was observed in 83% of clinical isolates and 16% of environmental isolates.

It was found that 74% of clinical isolates and 19% of environmental isolates carried tdh, whilst 26% of clinical and 22% of environmental isolates possessed trh.

3-3-Cytotoxicity. The ECPs obtained from clinical isolates all showed marked cytotoxicity towards CHO-K1 cells (mean LDH release 59.6%), whereas all but a few environmental isolates had very low cytotoxicity (11.9%) (Table 1 and Fig 2). Analysis of variance showed that the difference between clinical and environmental strains was significant at a P value of < 0.05.
Table (1): Putative virulence factors and genes, plasmid possession, and cytotoxicity of *Vibrio parahaemolyticus* isolates

| S train | Obtained from | Type and source (where known) | Virulence Characteristics and virulence genes*2 | P lasmid | Cytotoxicity |
|---------|---------------|-------------------------------|-----------------------------------------------|-----------|-------------|
|         |               |                               | P re | L ip | P ro | P PL | P dh | rh | DNA | LDH release*3 | Percent |
| R 1     | PHL S         | Clinical, NCIMB Reference Strain, UK | +   | -   | -   | +   |     | +  |     | 0.53 ± 0.01 | 62.0     |
| C 1     | NSVS          | Clinical, Norway              | +   | -   | +   | +   |     | +  |     | 0.48 ± 0.03 | 45.0     |
| C 2     | NSVS          | Clinical, Norway              | +   | -   | +   | +   |     | -  |     | 0.56 ± 0.01 | 71.9     |
| C 3     | NSVS          | Clinical, Norway              | +   | -   | -   | +   |     | -  |     | 0.53 ± 0.02 | 62.7     |
| C 4     | NSVS          | Clinical, Norway              | +   | +   | +   | +   |     | -  |     | 0.55 ± 0.002 | 67.0     |
| C 5     | NSVS          | Clinical, Norway              | +   | -   | +   | +   |     | -  |     | 0.55 ± 0.009 | 67.0     |
| C 6     | NSVS          | Clinical, Norway              | +   | +   | +   | +   |     | -  |     | 0.53 ± 0.02 | 61.0     |
| C 7     | NSVS          | Clinical, Norway              | +   | -   | +   | +   |     | +  |     | 0.53 ± 0.007 | 62.5     |
| C 8     | NSVS          | Clinical, Norway              | +   | -   | +   | +   |     | -  |     | 0.53 ± 0.002 | 63.0     |
| C 9     | NSVS          | Clinical, Norway              | +   | -   | +   | +   |     | -  |     | 0.51 ± 0.02 | 55.0     |
| C 10    | NSVS          | Clinical, Norway              | +   | +   | +   | +   |     | -  |     | 0.48 ± 0.03 | 47.0     |
| C 11    | KUM S         | Clinical, Japan               | +   | -   | -   | +   |     | -  |     | 0.54 ± 0.009 | 66.6     |
| C 12    | KUM S         | Clinical, Japan               | +   | +   | +   | +   |     | -  |     | 0.54 ± 0.008 | 66.0     |
| C 13    | KUM S         | Clinical, Japan               | +   | -   | +   | +   |     | -  |     | 0.51 ± 0.01 | 54.5     |
| C 14    | KUM S         | Clinical, Japan               | +   | -   | -   | +   |     | +  |     | 0.52 ± 0.03 | 58.0     |
| C 15    | PHL S         | Clinical, UK                  | +   | +   | +   | +   |     | -  |     | 0.54 ± 0.009 | 66.0     |
| C 16    | USC           | Clinical, Spain               | +   | -   | +   | +   |     | +  |     | 0.59 ± 0.01 | 51.7     |
| C 17 | USC | Clinical, Spain | + | - | + | - | + | 0.50 ± 0.01 | 52.0 |
| C 18 | USC | Clinical, Spain | + | - | + | + | - | 0.53 ± 0.004 | 61.0 |
| C 19 | USC | Clinical, Spain | + | + | + | + | - | 0.54 ± 0.006 | 64.0 |
| C 20 | USC | Clinical, Spain | + | - | + | - | - | 0.52 ± 0.005 | 60.5 |
| C 21 | CEF AS | Clinical, Italy | + | - | + | - | + | 0.50 ± 0.06 | 52.0 |
| C 22 | CEF AS | Clinical, UK | + | - | + | - | - | 0.49 ± 0.009 | 51.0 |
| R 2 | PHL S | Environmental, NCTC Reference Strain, UK | + | - | + | + | - | 0.46 ± 0.04 | 40.0 |
| E 1 | CEF AS | Environmental, Spain | - | - | - | - | + | 0.34 ± 0.004 | 5.0 |
| E 2 | CEF AS | Environmental, Spain | - | - | + | - | + | 0.34 ± 0.005 | 4.7 |
| E 3 | CEF AS | Environmental, Spain | - | - | + | + | - | 0.48 ± 0.04 | 46.8 |
| E 4 | CEF AS | Environmental, Spain | - | - | - | - | - | 0.34 ± 0.004 | 4.9 |
| E 5 | CEF AS | Environmental, Spain | - | - | - | + | + | 0.34 ± 0.007 | 3.0 |
| E 6 | CEF AS | Environmental, Spain | - | - | - | - | - | 0.34 ± 0.005 | 4.0 |
| E 7 | PHL S | Environmental, UK | - | - | + | - | + | 0.36 ± 0.04 | 10.0 |
| E 8 | PHL S | Environmental, UK | - | - | - | - | - | 0.34 ± 0.005 | 5.0 |
| E 9 | CEF AS | Environmental, UK | - | - | - | - | + | 0.53 ± 0.004 | 62.0 |
| E 10 | CEF AS | Environmental, UK | - | - | - | - | + | 0.34 ± 0.005 | 3.0 |
| E 11 | CEF AS | Environmental, UK | - | - | + | - | - | 0.34 ± 0.004 | 3.0 |
| E 12 | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.006 | 3.0 |
| E 13 | CEF AS | Environmental, UK | + | - | + | - | - | 0.34 ± 0.002 | 5.7 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.003 | 4.0 |
|----|-------|-------------------|---|---|---|---|---|----------------|-----|
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.36 ± 0.04   | 9.8 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.38 ± 0.05   | 15.8 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.007  | 4.8 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.36 ± 0.02   | 11.0 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.001  | 4.0 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.005  | 5.0 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.001  | 4.0 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.001  | 5.0 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.37 ± 0.02   | 13.7 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.003  | 4.5 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.37 ± 0.03   | 12.0 |
| E  | CEF AS | Environmental, Portugal | - | - | - | - | - | 0.34 ± 0.004  | 5.0 |
| E  | CEF AS | Environmental, Portugal | - | - | - | - | - | 0.37 ± 0.01   | 13.9 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.34 ± 0.002  | 5.0 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.38 ± 0.05   | 15.8 |
| E  | CEF AS | Environmental, UK | - | - | - | - | - | 0.35 ± 0.01   | 5.8 |

(1) CEFAS, Centre for Environment, Fisheries and Aquaculture Science, Weymouth, UK; PHLS, Public Health Laboratory Service, Southampton, UK (now Health Protection Agency); KUMS, Kyoto University Medical School, Japan; NSVS, Norwegian School of Veterinary Science, Oslo, Norway; USC, Universidad de Santiago de Compostela, Spain. (2) Virulence characteristics, K: Kanagawa haemolysis, Ur: urease, Lip: lipase, Pro: protease and Ph: phospholipase activities, and virulence genes (tdh and trh). (3) The LDH values are averages ±
standard deviations for nine replicates, where per cent cytotoxicity = \((\text{OD sample} - \text{OD spontaneous}) - (\text{OD total} - \text{OD spontaneous})) \times 100\) (see Methods).

\[
\begin{array}{cccccccc}
M & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\
220 \text{kDa} & & & & & & & \\
100 \text{kDa} & & & & & & & \\
60 \text{kDa} & & & & & & & \\
45 \text{kDa} & & & & & & & \\
30 \text{kDa} & & & & & & & \\
20 \text{kDa} & & & & & & & \\
12 \text{kDa} & & & & & & & \\
8 \text{kDa} & & & & & & & \\
\end{array}
\]

Fig 1. SDS-PAGE of soluble whole cell proteins from \(V.\ parahaemolyticus\) isolates, M, protein marker (M.W. = 8 kDa-220 kDa), 1: C2, 2: C8, 3: C9, 4: E4, 5: E5, 6: E2, 7: E1.

Fig 2. Cytotoxic effects of ECPs of clinical and environmental isolates of \(V.\ parahaemolyticus\) on CHO-K2 cell line. The X-axis indicates strains in the order that they appear in Table 1. Error bars represent the standard error of the mean.

**4-Discussion.** When a bacterial species exists in both virulent and avirulent forms, (32) reported that plasmid that detected in Kanagawa haemolysin positive strains of \(V.\ parahaemolyticus\) could be involved in production of this haemolysin or other features that contributed with pathogenicity of these strains. As a start to this study, extracts of the \(V.\ parahaemolyticus\) isolates were screened for the presence of plasmids. It was found that the proportions of clinical and environmental isolates harbouring plasmids were similar. These results support those of (33) who found similar percentages of plasmid-bearing forms in clinical and environmental \(V.\ parahaemolyticus\) isolates in the Pacific Northwest, and
suggested that the presence of plasmids in this bacterium does not correlate with Kanagawa haemolysis.

Similarly, SDS-PAGE analysis of whole cell soluble protein profiles showed no differentiation of clinical and environmental isolates.

A future line of research could investigate whether virulent *V. parahaemolyticus* isolates possess lysogenic bacteriophage that encoded virulence factors, as for example, occurs with lysogenic CTXφ bacteriophages carrying the cholera toxin that is found in O1 and O139 serotypes of *V. cholerae* (34). Phage’s have been found in *V. parahaemolyticus*. (15) and (35) have demonstrated that a filamentous phage f237 integrates into the chromosome of *V. parahaemolyticus*. The pattern of genomic organization of phage f237 is similar to that of the CTXφ phage.

Not all Kanagawa-positive isolates were positive for *tdh*, and two isolates that bore the *tdh* sequence were found to be negative for KP, conversely, 4 isolates that lacked the *tdh* amplicon produced KP; these results support the idea that the Kanagawa phenomenon is not always associated with expression of TDH encoded by the *tdh* gene and that such isolates still cause food borne illness (36, 37). Bej et al., (38) have previously reported that factors other than the presence of the *tdh* gene are involved in the haemolytic ability of *V. parahaemolyticus* (6, 39). However, the KP reaction has usually been used as a marker for detecting pathogenic *V. parahaemolyticus* (2). Nevertheless, Hondo et al., (10) have recorded that KP-negative strains may also be associated with gastroenteritis.

All isolates were tested on egg yolk plates and skim milk agar medium to test for phospholipase and protease activities, respectively; 19 (82.60%) clinical isolates of *V. parahaemolyticus* and 5 (15.62%) environmental isolates exhibited phospholipase activity; 19 (82.60%) clinical and 13 (40.62%) environmental showed protease activity. These findings suggest that mainly virulent forms of *V. parahaemolyticus* produce a phospholipase which may have a role in pathogenesis and is supported by the findings of Guhathakurta et al., (40) who demonstrated that phospholipase has a role as an important virulence factor in *V. parahaemolyticus* infection. The results for protease activity are less discriminating. However, other workers have reported that protease may have a role as a virulence factor in *V. parahaemolyticus* infection and may be involved in pathogenicity (40, 41). In the present study, the differences were significant at a *p* value of < 0.05 between both phospholipase and protease activities and cytotoxicity.

Lipase activity was also examined and exhibited in 6 (26%) clinical and 5 (15.62%) environmental isolates of *V. parahaemolyticus*; 19 isolates of *V. parahaemolyticus* showed high values for cytotoxicity in the absence of lipase activity (Table 1). The difference was not significant between lipase activity and cytotoxicity, suggesting that lipase does not play any role in pathogenicity in this bacterium.

The ability of *V. parahaemolyticus* isolates to hydrolyze urea has been used to predict potential pathogenicity of these bacteria (33, 42). In the current study urease activity of *V. parahaemolyticus* isolates was tested on urea agar base plates. All clinical isolates (100%) of *V. parahaemolyticus*, but only 46.87% (15/33) environmental isolates showed urease activity. Of the urease positive isolates, there were 4 (3 clinical and 1 environmental) having both *tdh* and *trh*, 20 (17 clinical and 3 environmental) having *tdh* only, 8 (3 clinical and 5
environmental) having \( trh \) only, and 10 (4 clinical and 6 environmental) had \( tdh \) or \( trh \) neither. Therefore, these results lead to a conclusion that the urease gene is not linked to the \( tdh \) or \( trh \), haemolysin genes. Park, (43) and Honda, et al., (44) also reported that no relationship exists with haemolysin genes and urease positive \( V. \) \textit{parahaemolyticus}. Osawa, et al., (45) also reported that there is no correlation between the presence \( tdh \) gene and urease activity, but it is possible to correlated with \( trh \) gene.

It was necessary, to determine the cytotoxicity characteristics of \( V. \) \textit{parahaemolyticus} isolated from clinical and environmental samples to understand their roles in gastroenteritis illnesses. It has demonstrated that a variety of \textit{Vibrio} species isolated from the environment and marine organisms secrete several ECPs with toxic effects which are classified into haemolysins and proteases. Cytotoxicity of ECP’s to CHO-K1 cells was performed to assess the potential virulence of both clinical and environmental isolates of \( V. \) \textit{parahaemolyticus}. The majority of clinical isolates show a higher level of cytotoxicity than the environmental isolates. A minority of environment isolates show the same levels of cytotoxicity as the proven virulent clinical isolates and these environmental isolates may be potentially virulent. Very similar, if not identical results were obtained with the same set of isolates whose ECP’s were tested for cytotoxicity against a colorectal human cell line (46). (47) have reported that the cytotoxicity of clinical isolates higher than environmental even though there is no statistical differences between them; and they suggested that there are other than TDH virulence factors, and the pathogenicity of this organism is not understood.

Prior to this study, certain activities such as haemolysis, protease, phospholipase and urease had been linked to the virulence of \( V. \) \textit{parahaemolyticus}. In the present study, these activities were screened and compared with the cytotoxicity of ECPs in clinical and environmental \( V. \) \textit{parahaemolyticus} isolates. Analysis of variance for \( V. \) \textit{parahaemolyticus} isolates showed that distribution of urease, protease, phospholipase and Kanagawa haemolysis activities were significantly related to cytotoxicity as assayed by CHO-K1 cells. Therefore, it may be possible that collectively these enzyme activities have a role in cytotoxicity. Lack of one or more of these components may still leave the strain virulent.

It can be seen that there is as yet no single factor which instils virulence on \( V. \) \textit{parahaemolyticus}. However, several polymerase chain reaction (PCR) methods are extensively used to allow for rapid detection of certain virulence genes (24, 38, 48) and new PCR markers have been recently found (46).

In conclusion, virulence was not associated with the presence of plasmids and whole cell protein profiles could not differentiate clinical and environmental isolates of this bacterium. However, a combination of enzyme activities may determine virulence but there is as yet no single marker or defined set of markers that can be used to detect all virulent forms of \( V. \) \textit{parahaemolyticus}. Further analysis of the exact nature of extracellular proteins produced by \( V. \) \textit{parahaemolyticus} may well unravel the virulence exhibited by this organism.

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