Molecular Characterisation of Clinical and Environmental Isolates of Vibrio parahaemolyticus

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This thesis is dedicated to my husband,

Mr Haidar Al-Zoamil and my son Ali
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Molecular Characterisation of Clinical and Environmental Isolates of *Vibrio parahaemolyticus*

By

Hadaf Mahdi Kadhim

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

Doctor of Philosophy

School of Biomedical and Biological Sciences
Faculty of Science

This project funded by Ministry of Higher Education
Baghdad, Iraq

December 2012
Molecular characterisation of clinical and environmental isolates of *Vibrio parahaemolyticus*

Hadaf Mahdi Kadhim

Abstract

The halophilic bacterium *Vibrio parahaemolyticus* is widely distributed as a natural inhabitant of marine and estuarine environments. Some strains cause gastroenteritis in humans. Clinical isolates are thought to possess virulence factors that are absent from the majority of environmental isolates. Studies were undertaken to differentiate clinical (virulent) and environmental (mainly avirulent) forms of *Vibrio parahaemolyticus*. Initially, identification and confirmation of a total of 55 *V. parahaemolyticus* isolates (23 clinical and 32 environmental) was carried out by using selective media, biochemical and nutritional tests. Identity was confirmed by specific polymerase chain reaction (PCR) targeting the *toxR* gene. In an attempt to differentiate between virulent and avirulent forms *V. parahaemolyticus*, potential virulence factors (enzyme activities), presence of plasmids and analyses of whole cell protein profiles and extracellular products (ECPs) by SDS-PAGE were performed. The results suggested that the presence of plasmids in isolates was not linked to virulence and SDS-PAGE profiles did not differentiate between virulent and avirulent forms, but a combination of enzyme activities may contribute to virulence. The ECPs of all 55 *V. parahaemolyticus* isolates were tested for their cytotoxicity towards two types of cell lines, the clinical isolates showed that 21 out of 23 (91%) and 2 out of 23 (8.69%) showed high and medium cytotoxicity, respectively. Amongst the environmental isolates 2 out of 32 (6.25%), 2 out of 32 (6.25%) and 28 out of 32 (87.5%) showed high, medium and low cytotoxicity, respectively. Randomly amplified polymorphic DNA (RAPD)-PCR was used to analyse the two groups of isolates. Firstly a 600 bp band was recognised in mainly clinical isolates. This DNA fragment was cloned and sequenced and found to code for an outer membrane protein (OMP). Two PCR primers were designed to specifically amplify a 200bp unique sequence from presumptive virulent strains (PCR-OMP); however, not all clinical isolates were positive (21 out of 23, 91%). A second RAPD-PCR identified a further unique band of approximately 310 bp in mostly clinical isolates. After cloning this band’s DNA, the DNA sequence revealed a hypothetical gene, *htp*, whose function is not known. Specific primers, VPHTP1 and VPHTP2 were developed for the detection of the *htp* sequence, but again not all clinical isolates were positive (19 out of 23, 82.6%). This led to the development of a multiplex M-PCR which detects all isolates of *V. parahaemolyticus* and differentiates them into potentially virulent and avirulent forms. The M-PCR works by targeting the *toxR* gene, and sequences for *omp* and *htp*. The M-PCR was performed on all *V. parahaemolyticus* isolates used in this study, as well as other *Vibrio* species and a selection of non-*Vibrio* species. The amplification of *toxR* gene 367 bp fragment was found in all *V. parahaemolyticus* tested; all clinical isolates (100%) showed amplification of *omp* and/or *htp*. This multiplex PCR detected 3 (9.37%) environment isolates, which may potentially be able to cause disease. No amplification was seen for the other species tested. Thus, the M-PCR could be used for identifying *V. parahaemolyticus* and detecting / differentiating potentially virulent and avirulent forms. This method should be useful for diagnostic purposes and epidemiological studies.
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Author’s Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. This study was financed with the aid of Ministry of the Higher Education and Scientific Research/Iraq. Relevant scientific seminars and conferences were attended at which work was presented and papers have been prepared for publication:

Publications


Other papers that will be submitted

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

Development of a multiplex polymerase chain reaction (M-PCR) for detection and identification of virulent and avirulent forms of Vibrio parahaemolyticus.

Oral presentation


Poster presentations


Randomly amplified polymorphic DNA (RAPD) method for differentiating clinical and environmental isolates of Vibrio parahaemolyticus. Vibrio 2010, Biloxi, Mississippi, USA, November 2010.

Molecular analysis for comparing between clinical and environmental isolates of *Vibrio parahaemolyticus*. Postgraduate Society Conference, Plymouth University, March 2011.

Use of cytotoxic effect of extracellular products (ECPs) of *Vibrio parahaemolyticus* on cell lines to distinguish virulent isolates from avirulent isolates. Centre for Research in Translational Biomedicine, Plymouth University, April 2011.


Cloning and sequence analysis of the gene that produce a RAPD-band unique to virulent *Vibrio parahaemolyticus*. SGM Autumn Conference 2011, Global disease burden of enterics Display, York, September 2011.

Polymerase chain reaction (PCR) method for rapid detection of virulent forms of *Vibrio parahaemolyticus*. *Vibrio* 2011, Santiago de Compostela, Spain, November 2011.

Development of polymerase chain reaction (PCR) for the detection of virulent forms of *Vibrio parahaemolyticus*. Centre for Research in Translational Biomedicine (CRTB), Plymouth University, July 2012.

**Conferences and workshops attended**

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Practical techniques in molecular biology workshop, Plymouth University-16th – 19th July 2012

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Date………………………………..
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<tr>
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<td>Caco-2</td>
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<td>thermostable direct haemolysin gene</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>thl</td>
<td>thermolabile haemolysin gene</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>toxR</td>
<td>ToxR transmembrane protein gene</td>
</tr>
<tr>
<td>trh</td>
<td>thermostable direct haemolysin related gene</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TSA</td>
<td>tryptone soya agar</td>
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<tr>
<td>TSB</td>
<td>tryptone soya broth</td>
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<tr>
<td>TTSS</td>
<td>type III secretion system</td>
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<td>Ure</td>
<td>urea hydrolysis</td>
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<td>UV</td>
<td>Ultra violet</td>
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<tr>
<td>V</td>
<td>volts</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indoyl-β-D-galactoside</td>
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</table>
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW
Chapter 1: Introduction and Literature Review

1.1 Introduction

*Vibrio parahaemolyticus* is a natural halophilic bacterial inhabitant of the marine environment. It causes food borne illness (gastroenteritis) and is attracting increasing interest worldwide where raw or undercooked seafood is often consumed (Chao et al., 2009). It has been found to be widely distributed in coastal waters in temperate zones and can be readily isolated from seafood (Honda and Iida, 1993). Not all *V. parahaemolyticus* strains are considered to be pathogenic. 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.

The aim of this chapter is to review the characteristics of *V. parahaemolyticus* and other human pathogenic *Vibrio* species, review the virulence properties of *V. parahaemolyticus*, and review the techniques, including molecular typing methods for the detection and identification of this bacterium.

1.2 Characteristics of *V. parahaemolyticus*

*V. parahaemolyticus* is a member of the family *Vibrionaceae*. The genus *Vibrio* includes 11 species of clinical importance (Janda et al., 1988). These species have been reported to be associated with human infection by consumption of contaminated food, untreated water or by contact directly with these species through skin wounds (Table 1.1).

*V. parahaemolyticus* causes seafood-borne disease (Feldhusen, 2000, Boyd et al., 2008). This bacterium is a Gram-negative, short, slender, curved rod. It is non-spore forming, and requires salt for optimal growth (Hayes, 1985). It causes
gastroenteritis, which is characterised by diarrhoea, vomiting, headache and abdominal cramps after consumption of contaminated food or water (Eley, 1992). This illness is often not treated and there is no vaccine available (Black, 1999). However, virulent strains of *V. parahaemolyticus* are often susceptible to antibiotics or the disease can be treated with oral rehydration alone (Daniels et al., 2000b). This organism also infects skin wounds of people who are exposed to contaminated water (Black, 1999, Miyoshi et al., 2008). It can cause septicemia particularly in those that are immunocompromised (Daniels et al., 2000a). *V. parahaemolyticus* multiplies very rapidly, therefore preventing *V. parahaemolyticus* gastroenteritis is difficult, as contaminated seafood may contain relatively large numbers of the bacteria after a short time (Ingraham and Ingraham, 1995).
Table 1.1 *Vibrio* species pathogenic for humans\(^1\)

<table>
<thead>
<tr>
<th>Species</th>
<th>Key features</th>
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<tbody>
<tr>
<td>1- <em>V. cholerae</em></td>
<td></td>
</tr>
<tr>
<td>a. (O1/O139 serotypes)</td>
<td>Cause of classical cholerae</td>
</tr>
<tr>
<td>b. (non O1/non O139 serotypes)</td>
<td>Wound infection and gastroenteritis</td>
</tr>
<tr>
<td>2- Virulent strains of <em>V. parahaemolyticus</em></td>
<td>Gastroenteritis and wound infection</td>
</tr>
<tr>
<td>3- <em>V. vulnificus</em></td>
<td>Primary septicemia and wound infection</td>
</tr>
<tr>
<td>4- <em>V. alginolyticus</em></td>
<td>Ear infection and wound infection</td>
</tr>
<tr>
<td>5- <em>V. mimicus</em></td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>6- <em>V. fluvialis</em></td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>7- <em>V. furnissii</em></td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>8- <em>V. hollisae</em></td>
<td>Gastroenteritis and septicemia</td>
</tr>
<tr>
<td>9- <em>V. metschnikovii</em></td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>10- <em>V. damsela</em></td>
<td>Wound infection</td>
</tr>
<tr>
<td>11- <em>V. cincinnatiensis</em></td>
<td>Septicemia</td>
</tr>
</tbody>
</table>

\(^1\)Data from Janda et al., (1988).
1.3 Genome analysis of *V. parahaemolyticus*

Genomic and comparative analyses of vibrios have revealed that vibrios possess two distinct chromosomes, confirmed by the whole genome sequencing of *V. cholerae* (Heidelberg et al., 2000), *V. parahaemolyticus* (Makino et al., 2003) and *V. vulnificus* (Chen et al., 2003). Furthermore, Okada et al., (2005) have demonstrated that vibrios commonly have two chromosomes. Among the vibrios, the size of the large chromosomes is relatively constant from 3.0 to 3.3 Mb; while the size of the small chromosomes varies from 0.8 to 2.4 Mb; and it is suggested that the small chromosome seems to be structurally more flexible than the large one. Essential genes are present on both chromosomes (Heidelberg et al., 2000, Makino et al., 2003).

Prior to genomic sequencing, genome analysis of *V. parahaemolyticus* showed that *V. parahaemolyticus* possess two circular chromosomes of approximately 3.2 and 1.9 Mb as characterised by pulse-field gel electrophoresis of whole genomic DNA (Yamaichi et al., 1999).

The whole genome sequencing of clinical strain RIMD2210633 confirmed that *V. parahaemolyticus* contained two circular chromosomes [chromosome 1 (3,288,558 bp) is larger than chromosome 2 (1,877,212 bp)] and showed that *V. parahaemolyticus* harboured genes for type three secretion systems clustered together with pathogenicity islands and virulence effectors. A G+C content of 45.4% for each chromosome, and ten copies of rRNA operons were found on chromosome 1 while only one copy on chromosome 2 (Makino et al., 2003).
1.4 Human pathogenic *Vibrio* species

*Vibrio* species are important bacteria that are extensively distributed in the marine environment, and pathogenic strains are of worldwide concern for the seafood industry (Jones et al., 2012). *V. parahaemolyticus*, toxigenic strains of *V. cholerae* (O1 and O139 serotype), and *V. vulnificus* are considered the main pathogenic *Vibrio* species that cause human disease. These bacteria are natural members of the marine environment and only certain virulent forms of these bacteria are responsible for human diseases. Other vibrios that cause human infection include *V. mimicus* and *V. alginolyticus* (Thompson et al., 2006).

Makino et al., (2003) have compared the genome of *V. parahaemolyticus* with the genome of *V. cholerae* and reported that the size of chromosome 1, 3.3 and 3.0 Mb respectively, does not differ much between of them, whereas chromosome 2 in *V. parahaemolyticus* (1.9 Mb) is much larger than in *V. cholerae* (1.1 Mb) (Heidelberg et al., 2000, Makino et al., 2003). The number of unique genes on chromosome 2 of *V. parahaemolyticus* (56.8%) and *V. cholerae* (41.9%) is higher than that found in chromosome 1 of *V. parahaemolyticus* (29.5%) and *V. cholerae* (16.2%) suggesting that chromosome 2 is more different in structure and gene content than chromosome 1 (Makino et al., 2003). Serotypes O1 and O139 of *V. cholerae* cause cholera that is characterised by dehydration because of the passage of voluminous watery stools, hypovolemic shock, and acidosis; and without prompt treatment can cause death (Kaper et al., 1995). These *V. cholerae* bacteria produce cholera toxin which is encoded by the lysogenic bacteriophage CTXΦ (Waldor and Mekalanos, 1996).
Chapter 1

**V. vulnificus** is found in temperate estuarine ecosystems throughout the world. This bacterium is similar phenotypically to **V. parahaemolyticus**; however, based on lactose fermentation via $\beta$-D- galactosidase production, it can be distinguished from **V. parahaemolyticus** (Hollis et al., 1976). This organism causes wound infections through contact with contaminated estuarine waters (Smith and Oliver, 2006). **V. vulnificus** also causes gastroenteritis and septicemia following consumption of contaminated seafood, especially raw oysters by immunocompromised people (Cañigral et al., 2010). It has a number of virulence factors including haemolysin which is a heat labile lytic enzyme (Bang et al., 1999); capsular polysaccharide (Wright et al., 1990), protease (Miyoshi et al., 1987), cytolysin (Gray and Kreger, 1985), phospholipase (Testa et al., 1984) and siderophore (Okujo and Yamamoto, 1994).

**V. alginolyticus** has been found in seawater worldwide and causes wound infection (Spark et al., 1979). It is isolated from marine organisms as part of their saprophytic microbiota (Carli et al., 1993). Most **V. alginolyticus** infections are self-limiting and opportunistic. Zen-Yoji et al., (1973) first characterised **V. alginolyticus** as a human pathogen. Both virulent and avirulent forms of **V. alginolyticus** exist; the virulent forms cause disease in both human and certain marine organisms (Cai et al., 2009). Furthermore, it is also potentially pathogenic to shellfish, such as shrimp (Wang and Chen, 2005), and fish (Balebona et al., 1998). **V. alginolyticus** is phenotypically close to **V. parahaemolyticus**; however, sucrose fermentation and other phenotypic characteristics can be used to distinguish it from **V. parahaemolyticus** (Hollis et al., 1976). Blake et al., (1979) have used four biochemical tests (fermentation of sucrose and lactose, tolerance to NaCl and Voges-Proskauer test) to distinguish **V. alginolyticus**, **V. parahaemolyticus** and **V. vulnificus**.


V. *mimicus* is frequently distributed in estuarine environments and fresh water. *V. mimicus* and *V. cholerae* O1 and O139 serotypes are similar in most biochemical pathways and some O antigens; but differ in sucrose fermentation on thiosulfate/citrate/bile salt/sucrose agar (TCBS), *V. cholerae* exhibit sucrose positive (yellow) colonies, where *V. mimicus* strains exhibit sucrose negative (green to blue) colonies (Davis et al., 1981). Virulence genes detected in some *V. mimicus* clinical strains are found to be similar to those present in heat-stable enterotoxin producing *Escherichia coli* (Shi et al., 1998), and homologous to the cholera toxin and enterotoxin genes in *V. cholerae* (Shi et al., 1998); and the *tdh* gene in *V. parahaemolyticus* (Terai et al., 1990).

*V. fluvialis* causes gastroenteritis after consumption of raw or undercooked seafood, it was first isolated and identified in 1975 in a patient with diarrhoea in Bahrain (Furniss et al., 1977). Between 1976 and 1977, this bacterium caused an outbreak of diarrhoea in more than 500 patients in Bangladesh (Huq et al., 1980). *V. fluvialis* also caused an outbreak of gastroenteritis in India in 1981 (Thekdi et al., 1990). It has also been isolated from seafood, water, aquatic animals, sediment and from human with diarrhea (Pruzzo et al., 2005). *V. fluvialis* produces metalloprotease similar to that of *V. vulnificus* (Miyoshi et al., 2002). Furthermore, Lockwood et al., (1982) showed that this bacterium produces four factors including a factor that elongates Chinese hamster ovary (CHO) cells, a cytolysin (haemolytic factor), non-haemolytic CHO cell-killing factor, and a protease.

*V. furnissii* was described in 1983 and classified as an aerogenic biogroup of *V. fluvialis* that produced gas from glucose. Based on genetic analysis, *V. furnissii* was found to be as a new species of *Vibrio*. The isolation frequency of *V. furnissii* is less than that for *V. fluvialis*, however, it has often been isolated from
environmental and rarely cases of gastroenteritis (Brenner et al., 1983). It has been isolated from stool of patient with diarrhoea (Hickman-Brenner et al., 1984). Furthermore, Dalsgaard et al., (1997) have reported that V. furnissii has been isolated from 14 patients (6 with diarrhoea and 8 without symptoms) during a cholera surveillance program in Peru. The virulence of this bacterium is associated with a haemolytic factor; and extracellular products were shown to damage HeLa cells (Magalhães et al., 1993). Phosphomannomutase (PPM) also plays a role in virulence of V. furnissii inducing intestinal damage in mice after oral uptake (Kim et al., 2003a).

V. hollisae is associated with consumption of raw seafood (Carnahan et al., 1994). V. hollisae commonly cause gastroenteritis but rare of cases of septicaemia and wound infection (Hickman et al., 1982, Hlady and Klontz, 1996). This bacterium produces a haemolysin similar to that of the TDH of V. parahaemolyticus (Yoh et al., 1986, Nishibuchi et al., 1988). This haemolysin encoded by tdh gene that shows to be 93% identical to the tdh gene of V. parahaemolyticus (Yamasaki et al., 1991). Moreover, V. hollisae produces an extracellular toxin factor that elongates CHO cells and induces intestinal fluid accumulation in suckling mice.

V. metschnikovii is occasionally isolated from human cases but is frequently isolated from the environment. This bacterium is potentially pathogenic to larval giant clams with infection leading to general disintegration of the tissue and total mortality (Sutton and Garrick, 1993). It has been isolated from fish in Brazil (Matte et al., 2007). Dalsgaard et al., (1996) have detected plasmids in V. metschnikovii but their functions were not investigated. This bacterium produces alkaline serine proteases and a metalloprotease (Kwon et al., 1994, Kwon et al., 1995). V.
*metschnikovii* produces a cytolysin that is able to induce haemolysis of rabbit erythrocytes (Miyake et al., 1989).

*V. damsela* is a member of the microflora of healthy carcharhinid sharke (Pruzzo et al., 2005). *V. damsela* can cause fatal wound infection (Clarridge and Zighelboim-Daum, 1985). It has been recorded that this bacterium was responsible for two cases of fatal necrotising fasciitis in Japan (Yamane et al., 2004). This bacterium produces a cytolysin toxin involved in its pathogenicity. This toxin is cytotoxic to CHO cells and lethal to mice (Kreger, 1984, Kothary and Kreger, 1985). Extracellular products of *V. damsela* have been tested *in vivo* and found to exhibit phospholipase, haemolytic and cytotoxic activities (Fouz et al., 1993). A siderophore-mediated iron sequestering system is also considered as a virulence factor of *V. damsela* (Fouz et al., 1997).

*V. cincinnatiensis* is the most recently described *Vibrio* human pathogen in 1986, it was isolated from a case of septicemia and meningitis in a 70 year old patient (Bode et al., 1986). *V. cincinnatiensis* has infrequently been isolated from human, and the pathogenicity of this bacterium is still questionable (Nishibuchi, 2006). It has been also isolated from *Mytilus galloprovincialis* harvested from the Adriatic Sea in Italy (Ripabelli et al., 1999). Furthermore, this bacterium was found in fresh water of Ohta River in Japan and demonstrated haemolytic and cytotoxic activities (Venkateswaran et al., 1989).
1.5 The incidence and a brief history of *Vibrio parahaemolyticus* infections

1.5.1 The incidence in seafood

Many researchers worldwide are interested in the incidence of *V. parahaemolyticus* and identify the ecological factors that affect it including temperature, salinity, turbidity and chlorophyll *a* (Julie et al., 2010). Depaola et al., (2000) have demonstrated that warm temperature and low salinity have been found to influence the incidence of this bacterium in North America when the authors collected the samples during the summer where the temperature and salinity of the seawater ranged from 27.8 to 31.7°C and 14.9 to 29.3 parts per thousand (ppt), respectively. Moreover, the highest incidence of total *V. parahaemolyticus* in Alabama oysters was in May and June with water temperature over 25 and salinity 17 ppt (Depaola et al., 2003a).

Furthermore, this organism has been mainly found in temperate water regions (Duan and Su, 2005a). Kaneko and Cowell, (1973) recorded that much higher levels of *V. parahaemolyticus* were detected in samples of oyster, water and sediment in the Chesapeake Bay (USA Atlantic coast) within the summer months, while very low levels were detected in these samples during the winter. Duan and Su., (2005a) have reported that when the water temperature is low, the sediment provides a protective environment for the survival of this bacterium. The densities of *V. parahaemolyticus* in oyster were positively associated with water temperature, turbidity and dissolved oxygen (Parveen et al., 2008). When water is more turbid and richer in organic matter this leads to an increase in the presence of bacteria (Parveen et al., 2008). However, turbidity is less important than temperature, with the detection of *V. parahaemolyticus* in oysters harvested from warm waters more likely than those from cold water (Kaysner and Depaola, 2000).
In Europe, the increasing incidence of *V. parahaemolyticus* infection has been found to be attributed to the emergence of the O3:K6 serotype, for example, the O3:K6 serotype was related to an outbreak in Spain (Martinez-Urtaza et al., 2005), and the authors reported that the emergence of O3:K6 pandemic strains in Europe is a public health concern.

### 1.5.2 Outbreaks of illness

*V. parahaemolyticus* gastroenteritis was first described in Japan, in 1951 (Eley, 1992). Since then, cases of food poisoning caused by *V. parahaemolyticus* have been reported from many maritime Asian countries, including India (Yeung and Boor, 2004, Alagappan et al., 2010), China (Wang et al., 2007, Liu et al., 2009, Chen et al., 2012), Korea (Lee et al., 2008, Jun et al., 2012), Japan (Alam et al., 2003, Kubota et al., 2011), Taiwan (Pan et al., 1997, Chang et al., 2011) and Thailand (Yamamoto et al., 2008, Jatapai et al., 2010). In Korea, Laos, and Indonesia, *V. parahaemolyticus* outbreaks are recorded as due to a pandemic strain (Matsumoto et al., 2000). In Japan, about 70% of gastroenteritis cases are due to *V. parahaemolyticus* (Kaneko and Colwell, 1973). In Taiwan, it caused outbreaks of food borne disease between 1986 to 1995 (Pan et al., 1997). In Iran, few studies on the incidence of this bacterium in seafood have been reported, for example, *V. parahaemolyticus* was isolated 9.3% from shrimp (Rahimi et al., 2010), and 11% from retail shrimp (Zarei et al., 2012).

*V. parahaemolyticus* causes infection in the United States, first reported in 1971 (Molenda et al., 1972), and a number of common source gastroenteritis outbreaks are attributed to this organism (Spite et al., 1978). Two outbreaks in Texas (416 cases) and in Washington (43 cases) were after consumption of raw oysters (Depaola et al., 2000). Mclaughlin et al., (2005) also described that *V.*
*parahaemolyticus* caused gastroenteritis related to the consumption of raw oysters (Su and Liu, 2007).

### 1.6 Prevalence and occurrence of *V. parahaemolyticus* in Europe

Food borne disease caused by *V. parahaemolyticus* is rarely identified in European countries. However, the increase in consumption of raw or undercooked seafood and the phenomenon of global warming and increased water temperature has led to increase concern about *V. parahaemolyticus* and the enhanced risk of *V. parahaemolyticus* infection. Hooper et al., (1974) recorded the first outbreak in the UK in 1974.

In Galicia, Northern Spain in 1999, an outbreak of 64 cases of *V. parahaemolyticus* gastroenteritis due to consumption of raw oysters was recorded (Lozano-León et al., 2003). In 2004, another outbreak of 80 cases in Spain related with consumption of boiled crab has been reported by Martinez-Urtaza et al., (2005). Pathogenic *V. parahaemolyticus* has also been detected in Italy (Di Pinto et al., 2008, Caburlotto et al., 2009, Ottaviani et al., 2010, Fabbro et al., 2010, Serracca et al., 2011), Denmark (Kristensen, 1974) and France (Hervio-Heath et al., 2002, Robert-Pillot et al., 2004).

Prevalence of potentially pathogenic *V. parahaemolyticus* in Chinese mitten crabs has been reported in England by Wagley et al., (2009). In Norway, the occurrence of potentially pathogenic *V. parahaemolyticus* in blue mussels has been also reported (Bauer et al., 2006).
1.7 Global climate change and *Vibrio* infections

Although, the risk of *V. parahaemolyticus* infections in Europe is considered to be low (Wagley et al., 2008), Baker-Austin et al., (2010) have reported that *V. parahaemolyticus* and *V. vulnificus* infections are increasing in Europe and likely to be related with regional climatic trends. The geographical range of vibrios may vary with climate change, leading to increase exposure and human risk of infection (Lipp et al., 2002). Studying the impact of climate change on human health and infectious diseases spreading is central (Cook, 1992). Disease risks caused by vibrios are increasing worldwide with global warming (Harvell et al., 2002). Indeed, consideration of the link between global warming and disease emergence caused by vibrios is interesting.

Global climate change especially increasing seawater temperature may play an important role in increasing the incidence of *Vibrio* infections, for example, increases in water temperature are significantly associated with cholera epidemics (Colwell, 1996). An increase of temperature (5°C) can be major factor with regards to the incidence of human infection and is related to a risk ratio for cholera infection (Huq et al., 2005). The highest densities of vibrios in seawater occur when the temperature ranged between 20°C and 30°C (Tantillo et al., 2004). Increasing temperatures are related to a higher risk of *V. parahaemolyticus* in water, and pathogenic strains of this bacterium have been detected in French coastal water (Robert-Pillot et al., 2004). Infection caused by *V. parahaemolyticus* was also reported in Spain (Martinez-Urtaza et al., 2008). In Israel, the increasing risk of *V. vulnificus* infection as a result of change in climate and altered the levels of *V. vulnificus* populations in the seawater has also been reported (Paz et al., 2007). In Germany, an increase in wound infection associated with *V. vulnificus* has been linked to contact with the Blatic sea as a result of warm water (Frank et
Furthermore, Fouz et al., (2006) have reported that infections by *V. vulnificus* have been spread in the summer of 2004 in Denmark.

For 25 years sea surface temperature has risen in UK waters (between 0.6 and 0.8 °C) per decade in the southern North Sea (Mccip, 2010). Increases in marine vibrios and incidences of their infections around the coastal North Sea have been associated significantly with increasing sea surface temperature (Vezzulli et al., 2012).

1.8 Serotypes of *Vibrio parahaemolyticus*

*V. parahaemolyticus* serotypes have been classified on the basis of the antigenic qualities of the somatic (O) and capsular (K) antigens. In Japan, specific antisera are used to differentiate between 13 O groups and 71 K types (Iguchi et al., 1995, Yeung and Boor, 2004). Historically, serotypes O4:K68, and O1: K untypeable (KUT) of *V. parahaemolyticus* cause disease, and are genetically related to the emerging O3:K6 serotype. Moreover, it has been found that they share similar pulsed field gel electrophoresis and ribotyping patterns (Chowdhury et al., 2000). The O3:K6 *V. parahaemolyticus* has been revealed as the main serotype in a 1996 outbreak in India (Okuda et al., 1997b). *V. parahaemolyticus* serotype O3:K6 has also caused epidemics of gastroenteritis in North America, Japan and Southeast Asia (Nasu et al., 2000). Furthermore, in the United States, three outbreaks have been recorded on the Gulf, Atlantic, and Pacific coasts, and molecular analysis of these strains shows that they may have diverged from O3:K6 strains (Chowdhury et al., 2000). Very recently, it was revealed that *V. parahaemolyticus* O3:K6 serotype caused about 79% of the cases of gastroenteritis from 2004 to 2010 in Sinaloa, Mexico (Velazquez-Roman et al., 2012). In Europe, the pandemic strains of *V. parahaemolyticus* O3:K6 serotype
was revealed to be present and persistent in French coastal areas (Quilici et al., 2005). Pandemic *V. parahaemolyticus* O3:K6 strains have been also detected in Italy (Ottaviani et al., 2008) and Russia (Nair et al., 2007). Furthermore, in Spain, Martinez-Urtaza et al., (2005) have reported that a unique clone of the virulent *V. parahaemolyticus* O4:K11 serotype has been found to be related with most clinical isolates and concurrent with the arrival of warmer tropical waters to the Galician coast.

### 1.9 Virulence factors and pathogenicity

Makino et al., (2003) have reported that more than 50 genes were identified in the genome sequence of *V. parahaemolyticus* that could possibly contribute to pathogenicity. These genes include those responsible for the biosynthesis of numerous pili, responsible for bacterial adherence and for numerous toxin homologues. The relationship between pathogenicity and the presence of these recently identified genes remains to be studied.

Virulence factors are considered to be the unique molecular features of a pathogen used to achieve colonisation, infection of the host and cause damage to a host. A comprehensive understanding of *V. parahaemolyticus* infection and what virulence factors are involved is still elusive. The following sections describe factors have been reported previously and are thought to contribute to the virulence and pathogenicity of *V. parahaemolyticus*.

#### 1.9.1 Thermostable direct haemolysin (TDH) and thermostable related haemolysin TRH

Virulent and avirulent forms of *V. parahaemolyticus* have been revealed by many studies. Most virulent strains of *V. parahaemolyticus* are reported to carry the *tdh* gene that encodes TDH or *trh* gene that codes for the TRH; some strains carry
V. parahaemolyticus isolates that carry both *tdh* and *trh* genes are strongly associated with clinical cases (Nishibuchi and Kaper, 1995). The presence of *V. parahaemolyticus* carrying *tdh* and/or *trh* pathogenicity genes in seafood has been considered as a public health risk (Zarei et al., 2012). Yamazaki et al., (2010) have reported that THD and TRH are main virulence factors in this bacterium. Furthermore, Wagley et al., (2009) have used TDH and TRH as putative pathogenicity markers to detect the pathogenic strains of *V. parahaemolyticus* in seafood. Both TDH and TRH are comprised of 165 amino acid residues and have about 67% identity in their amino acid sequences (Honda and Iida, 1993). Kamruzzaman et al., (2008) have reported that TDH is a marker of virulent strains of *V. parahaemolyticus*. It shows a beta-type haemolysis on Wagatsuma agar, called the Kanagawa phenomenon positive phenotype (KP\textsuperscript{+}) (Honda and Iida, 1993). Miyamoto et al., (1969) reported that only 1 to 2% of strains from non-clinical (environmental) sources are KP\textsuperscript{+}. However, Cabrera-Garcia et al., (2004) have reported that the presence of the *tdh* gene that correlates with KP\textsuperscript{+} phenotype has been reported in 9 % of environmental *V. parahaemolyticus* isolates around Mexico; and Cook et al., (2002) reported that only 6 % of isolates found in molluscan shellfish on the Atlantic and Gulf Coast were KP\textsuperscript{+}. The *tdh* gene was present in 10.5% of *V. parahaemolyticus* isolated from the environment (Rojas et al., 2011). However, Wagley et al., (2008) have detected *tdh* gene in 12% of the environmental isolates of *V. parahaemolyticus*. Furthermore, Velazquez-Roman et al., (2012) have reported that 52% (75 out of 144) of *V. parahaemolyticus* environmental isolates carry *tdh* and/or *trh* genes and are considered as pathogenic strains. The *tdh* gene has been detected in (81/97) clinical isolates and (10/45) isolates from sea fish and none from fresh water (Hongping et al., 2011).
TRH is associated with the Kanagawa phenomenon negative phenotype (KP⁻) and/or with urease positive phenotype (Kelly and Stroh, 1989). Honda et al., (1988) have reported that KP⁻ strains of this organism have caused food poisoning outbreaks. In Japan, Shirai et al., (1990) have reported that the presence of the trh gene has been demonstrated in 7% of environmental strains of *V. parahaemolyticus*. Furthermore, Caburlotto et al., (2009) have demonstrated that 6% of the environmental *V. parahaemolyticus* isolates around Italy carried trh gene. Recently, the trh gene has been detected in 12% of clinical and 3.6% of environmental isolates of *V. parahaemolyticus* in eastern China (Vongxay et al., 2008). In Spanish Mediterranean coast, Roque et al., (2009) have detected the virulent strains of *V. parahaemolyticus* that carry tdh and trh genes in cultured bivalves.

TDH and TRH biological activities include haemolytic character, cytotoxicity, enterotoxicity and cardiotoxicity (Shirai et al., 1990, Nishibuchi et al., 1992). Adherence to cultured cells has also been assessed to measure bacterial virulence abilities (Baffone et al., 2000). TDH is a homo-dimer protein that has a molecular mass of 46 kDa. It is a pore-forming toxin, which is capable of causing haemolysis of erythrocytes from a number of mammalian species (Fabbri et al., 1999). A series of experiments employing rabbit intestinal loop tests with TDH-producing or non-producing *V. parahaemolyticus* isolates have been used to study enterotoxicity of TDH. Only TDH-producing *V. parahaemolyticus* isolates can induce fluid accumulation (Honda and Iida, 1993).

The cytotoxicity of *V. parahaemolyticus* has been tested on different host cell lines, including human colorectal epithelial cells (Caco-2) (Raimondi et al., 2000), and human epithelial cells (HeLa) (Yeung et al., 2002). Yeung et al., (2002) have
reported that cytotoxicity and adherence of \textit{V. parahaemolyticus} O3:K6 isolates to human epithelial (HeLa) cells was shown to be statistically higher than that for non-O3:K6 isolates.

TDH and TRH have been studied and considered as virulence factors to detect the pathogenic \textit{V. parahaemolyticus} isolates (Kaneko and Colwell, 1973, Matsuda et al., 2010). However, isolates lacking TDH and/or TRH still play a role in pathogenicity of \textit{V. parahaemolyticus}. Virulence determinants other than TDH and TRH in \textit{V. parahaemolyticus} strains remain to be investigated (Makino et al., 2003, Lynch et al., 2005, Vongxay et al., 2008).

### 1.9.2 Enzyme activity

Urease is defined as an enzyme from bacteria, fungi and plants that catalyses the hydrolysis of urea to carbon dioxide and ammonia (Olivera-Severo et al., 2006). The ability to hydrolyse urea can be used to predict potential pathogenicity of bacteria and hence the urease enzyme may be an important virulence marker for virulent \textit{V. parahaemolyticus} strains (Kelly and Stroh, 1989). Urease activity is associated with the presence of the \textit{trh} gene (Okuda et al., 1997a). It has been purified from culture supernatant of KP negative, urease positive \textit{V. parahaemolyticus} isolates by Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), and has a high molecular weight of 275 kDa, containing three subunits of 33 kDa, 59 kDa and 85 kDa (Cai and Ni, 1996). Cai and Ni, (1996) studied its contribution to pathogenicity showing that it can cause intestinal fluid accumulation and was considered an important marker of pathogenesis. However, pandemic O3:K6 strains that were isolated from patients were found to be urease negative and the link between pathogenesis and urease was unclear (Yeung and Boor, 2004). Urease producing can be involved in the
pathogenicity of shiga toxin-producing *Escherichia coli* (Steyert and Kaper, 2012). Furthermore, urease has been considered as an important pathogenic factor for *Proteus mirabilis* (Jones et al., 1990) and *Helicobacter pylori* (Eaton et al., 1991).

Virulent strains of *V. parahaemolyticus* produce several extracellular proteases, such as a 62 kDa zinc metalloprotease (PrtV) (Lee et al., 1995b, Yu and Lee, 1999). Indeed, extracellular proteases present in the extracellular products (ECPs) and the possible role of proteases as virulence determinants of *V. parahaemolyticus* to tiger prawn have been reported by Sudheesh and Xu, (2001). Moreover, a serine protease has been detected in the extracellular proteins of *V. parahaemolyticus* which is a monomeric protein that has a molecular mass of 43 kDa, and recognised as one of a number of putative virulence factors associated with pathogenesis. It was purified from a clinical isolate that was negative for *tdh* and *trh* genes and has been found to exert a cytotoxic effect on Chinese hamster ovary, HeLa, Vero, and Caco-2 cells, and caused lysis of erythrocytes leading to haemorrhage and death in mice (Lee et al., 2002a). More recently, Liu et al., (2011) have purified and identified a new extracellular serine protease (63 kDa) from a pathogenic strain of *V. parahaemolyticus* and found it to be toxic to zebra fish. Furthermore, proteases were found to play an important role in the virulence of clinical isolates of *V. parahaemolyticus* lacking *tdh* and *trh* genes and were shown to exhibit cytotoxic activity on Vero and CHO-K1 cells (Ottaviani et al., 2005). Recently, Chakraborty and Surendran (2009) concluded that not only TDH and TRH, but also proteases, play a main role in the pathogenicity of *V. parahaemolyticus* and contribute to enteropathogenicity. In addition, a *V. parahaemolyticus* zinc metalloprotease (VppC, 87 kDa) was also found in ECPs of virulent *V. parahaemolyticus* strains and is thought to play a role in fish pathogenicity (Kim et al., 2002, Luan et al., 2007). *V. vulnificus* also produces
protease, it has been purified and characterised by Miyoshi et al., (1987) who reported that the protease have caseinolytic, elastolytic and collagenolytic activities. Nottage and Birkbeck, (1987) have reported that the proteinase of *V. alginolyticus* contributes to pathogenesis of bivalve vibriosis. Furthermore, a cysteine protease has been demonstrated as important pathogenicity factor in *Vibrio harveyi*, and is considered a major exotoxin affecting the tiger prawn (Liu and Lee, 1999).

Phospholipases can be virulence factors for some bacterial species; these species cause infection and massive tissue destruction, such as skin and lung infections caused by *Pseudomonas aeruginosa*. Many other bacteria secrete phospholipase that is cytolytic and causes tissue damage within infection (Songer, 1997). Phospholipases have been considered as a virulence factor in *V. parahaemolyticus* and are associated with pathogenicity (Guhathakurta et al., 1988). The relationship between haemolytic and phospholipase activity has been demonstrated in some vibrios such as toxigenic *V. cholerae* O139 whereby the haemolysin exhibited phospholipase activity (Pal et al., 1997). Furthermore, the haemolysin of *V. harveyi* strongly correlated with phospholipase activity (Zhong et al., 2006). Lee et al., (2002b) have reported that the phospholipase of *V. mimicus* showed haemolytic activity. Phospholipase plays a role as a putative virulence factor in *V. vulnificus* (Koo et al., 2007). Furthermore, phospholipase is one of the virulence factors that contribute to pathogenicity of *V. harveyi* in the tiger prawn (Liu et al., 1996).
1.9.3 Adherence and colonisation factors

Adherence and colonisation factors of enteropathogenic bacteria play an important role in the colonisation of the host gut epithelial surface (Dai et al., 1992). Yeung et al., (2002) have hypothesised that the positive motility phenotype of *V. parahaemolyticus* O3:K6 isolates correlated with virulence. Nakasone and Lwanaga, (1990) have purified pili of *V. parahaemolyticus* from a KP+ strain and found them to be adhesive to the rabbit intestine, and they suggested that pili of this bacterium play an important role in colonisation.

*V. parahaemolyticus* is motile due to possessing both polar and lateral flagella that enable it to swim and swarm, respectively (Mccarter and Silverman, 1990, Stewart and Mccarter, 2003). Genes on chromosome 1 or 2 of *V. parahaemolyticus* are found to be responsible for biosynthesis of the polar or lateral flagella, respectively. Zorrilla et al., (2003) have reported that swarming could be used as a marker to distinguish the virulent forms of *V. harveyi* and *V. parahaemolyticus* isolates. Moreover, the lateral flagellum has been demonstrated as an important colonisation factor.

*V. parahaemolyticus* has recently been shown to possess a capsule that may contribute to pathogenicity (Broberg et al., 2011). The capsular polysaccharide of *V. vulnificus* is considered a virulence factor and it is important for colonisation, uptake and survival of this bacterium in oysters (Srivastava et al., 2009). A capsule also acts as a virulence factor in *V. cholerae* O139 and plays a role in intestinal colonisation (Waldor et al., 1994), adherence (Yamamoto et al., 1994) and makes the bacteria partially resistant to phagocytosis (Albert et al., 1999).

Another potential pathogenicity determinant of *V. parahaemolyticus* is the siderophore (Tanabe et al., 2003) which is a low molecular mass microbial
compound with a very high affinity for binding iron (Winkelmann, 2002). Dai et al., (1992) have reported that the siderophore and two higher molecular mass (77 and 80 kDa) outer membrane proteins (OMPs) could play a role as virulence factors since Iron is necessary in the pathogenesis of *V. parahaemolyticus*. Production of a siderophore is significantly enhanced with Iron limited *in vitro* culture. Siderophores are necessary for infection in other human pathogens such as *Burkholderia, Pseudomonas* and mycobacteria (Ratledge and Dover, 2000). Siderophores may be important to the virulence of a number of enteropathogenic bacteria (Dai et al., 1992).

### 1.9.4 Mobile genetic elements

Bacteriophages are generally isolated from the ecosystem which is the habitat for the host strains of bacteria (Baross et al., 1978, Shivu et al., 2007). Phages are present among *V. parahaemolyticus* bacteria. Two filamentous phages Vf12 and Vf33 have been found in *V. parahaemolyticus* strains (Chang et al., 1998). Nevertheless, these two phages do not bear the *tdh* and *trh* genes, but the genome of these phages can integrate into plasmids or chromosomal DNA of *V. parahaemolyticus* and other *Vibrio* species. Therefore, these filamentous phages could contribute to the mobilisation of virulence genes (Chang et al., 1998).

Furthermore, a filamentous phage, f237, from O3:K6 *V. parahaemolyticus* has been isolated by Nasu et al., (2000). The open reading frame, ORF8 in this phage is not found in other filamentous phages. Consequently, the *orf8* gene has been demonstrated as a genetic marker for pandemic O3:K6 strains (Iida et al., 2001).

Lysogenic CTXø bacteriophage carry a cholera toxin that is found in O1 and O139 serotypes of *V. cholerae* (Waldor and Mekalanos, 1996, Faruque et al., 1998). The pattern of genomic organisation of phage f237 is similar to that of CTXø phage;
both of these phages are single stranded DNA and integrate into the genome of bacteria. Emergence of new pathogenic strains of *V. parahaemolyticus* may result from exchange of mobile genetic elements (Chang et al., 1998). The O4:K68 serotype of *V. parahaemolyticus* has filamentous phage, Vf O4:K68 (Chang et al., 2002). Seguritan, et al., (2003) have isolated two phages, VP16T and VP16C from an environmental isolate of *V. parahaemolyticus*. The gene structures and the distribution among *Vibrio* species of Vf12 and Vf33 have been analysed by Chang, et al., (1998).

Interestingly, phages carry out a process known as lysogenic conversion that plays a role in the virulence of pathogenic *V. harveyi*; this bacterium is found in the intestinal tract of marine fauna and in tropical warm marine environments (Oakey and Owens, 2000). Strains of this bacterium have caused significant mortalities in the shrimp culture industry worldwide (Alabi et al., 1999). *V. harveyi* myovirus like virus (VHML) is a phage that is able to infect a narrow host range and has shown a preference for certain strains of *V. harveyi* (Oakey and Owens, 2000). The infection of VHML phage in *V. harveyi* strains cause up-regulation of protein excretion, and up-regulation of haemolysin production and a rise in virulence towards prawns. Moreover, the existence of this phage may confer virulence to *V. harveyi* strain 642, that has been studied by Munro et al., (2003).

A plasmid is an extrachromosomal covalently closed deoxyribonucleic acid molecule that can range in size from 2 Kbp – 200/300 Kbp (Kado, 1998). The genes carried on a plasmid tend not to be essential for the existence of the bacteria, but can convey a useful characteristic to the host bacterium (Brown, 2001). The copy number of a plasmid is the number of copies of a particular plasmid per cell; this can vary between very high with hundreds of copies per cell.
to very low with only one or two copies per cell. By the host machinery, a plasmid can be replicated independently of the main bacterial genome as it possesses an origin of replication. Some plasmids (the episomes) can be replicated by integrating themselves into a bacterial chromosome and are replicated along with it, much like lysogenic phage (Brown, 2001). Guerry and Colwell, (1977) and Twedt, et al., (1981) have reported two plasmids of about 24 Mdal (36.5 Kbp) and 60 Mdal (91 Kbp) in KP+ strains of *V. parahaemolyticus* O3:K6 isolated from patients.

Insertion sequences are the simplest of the mobile DNA elements and are usually distributed between bacterial genomes (Mahillon and Chandler, 1998). Honda et al., (1988) have demonstrated that non-O1 *V. cholera*, *V. mimicus* and *V. hollisae* strains causing diarrhoea, produce haemolysins similar to TDH. Terai et al., (1991) have described the involvement of insertion sequences in the spread of the *tdh* gene among *Vibrio* species. The genome structure of *V. parahaemolyticus* can be changed by insertion sequences that lead to loss of main virulence features (Kamruzzaman et al., 2008, Kamruzzaman and Nishibuchi, 2008). Seven *tdh* genes and their flanking regions have been analysed by Terai et al., (1991); this analysis revealed insertion sequence like elements which are collectively called ISV_s for insertion sequences in vibrios or sequences that were derived from the genetic rearrangement of the ISV_s.

**1.9.5 Type III secretion system of *V. parahaemolyticus***

The type III secretion system (T3SS) is an apparatus which is capable of causing direct protein secretion into a host organism e.g. eukaryotic cell. The T3SS is a main virulence factor of pathogenic bacteria that cause gastroenteritis including *Salmonella*, *Shigella* and enteropathogenic *Escherichia coli* (Hueck, 1998).
Genomic sequencing of *V. parahaemolyticus* RIMD2210633 revealed two T3SSs, namely, T3SS1 and T3SS2 (Makino et al., 2003). Park et al., (2004a) have recorded that T3SS homologues are found in *V. parahaemolyticus* and other *Vibrio* species including *V. tubiashii* and *V. alginolyticus*.

Furthermore, *V. cholerae* non-O1 and non-O139 are shown to have genes for T3SS as determined by genomic characterisation (Dziejman et al., 2005). Henke and Bassler, (2004) characterised three type III secretion gene clusters in *V. harveyi* but their contribution to the pathogenesis of this bacterium is unknown.

Park et al., (2004a) have found the T3SS1 is located on the large chromosome of *V. parahaemolyticus* while T3SS2 is found on the small chromosome of this bacterium. T3SS1 plays a role in cytotoxic activity, while T3SS2 is responsible for enterotoxicity. T3SS1 is found in clinical and environmental *V. parahaemolyticus* strains (Thompson et al., 2006). T3SS2 has been identified only in clinical *V. parahaemolyticus* strains that are positive for KP reaction (Makino et al., 2003), and play a role in the pathogenicity to humans as reported by Hiyoshi et al., (2010). However, Caburlotto et al., (2010) have reported that a number of environmental strains isolated from the North Adriatic sea around Italy carry T3SS2 and tested their pathogenicity on Caco-2 cells; the results indicated that these strains are considered to be a risk for human health. However, Kodama et al., (2007) have suggested that there are other unknown effectors involved in the pathogenic mechanism of this organism.

**1.10 Identification and detection methods**

Development methods for the detection of *V. parahaemolyticus* in seafood samples are necessary to reduce the risk of this organism and to ensure the safety of foods (Hara-Kudo et al., 2001).
1.10.1 Traditional methods for identification

*V. parahaemolyticus* is halophilic, growing best with 3-6 % NaCl. This organism grows even at high pH 9.0. The optimum temperature for growth is 37°C and the temperature range of growth is 8-44 °C (Hayes, 1985). The growth of *V. parahaemolyticus* can be enriched with media such as alkaline peptone water at 37 °C (Dupray and Cormier, 1983).

The US Food and Drug Administration Bacterial Analytical Manual described the most probable number method (MPN) that is usually used for detection of *V. parahaemolyticus* (Elliot et al., 1995). However, this method is time-consuming and labour intensive (Su and Liu, 2007). The international standard ISO/TS 21872-1 has been used to detect *V. parahaemolyticus* in seafood, Rosec et al., (2012) have reported that this method failed to detect *V. parahaemolyticus* in 44% to 53% of the living bivalve molluscs where specific PCR based method detected the specific gene toxR of *V. parahaemolyticus*, the authors have suggested that the need for a revision of the ISO/TS 21872-1 standard.

Chun et al., (1974) prepared a medium containing NaCl, phosphate buffer and teepol. This enrichment medium is used to isolate *V. parahaemolyticus* from marine samples. Another medium less frequently used for isolating this organism, is mannitol salt agar (Carruthers and Kabat, 1976).

Since then, thiosulfate citrate bile salts sucrose agar (TCBS) has been conventionally used for isolating and enumerating this bacterium, however other enteropathogenic vibrios grow well on it (Adams and Moss, 2000). The quality and recovery of growth of *V. cholerae, V. fluvialis, V. parahaemolyticus* and *V. vulnificus* varied significantly on different brands of TCBS agar tested (West et al., 1982). However, it is not a differential medium for *V. parahaemolyticus*. A selective
medium was developed for the culture of *V. parahaemolyticus*, Hara-Kudo et al., (2001) and Blanco-Abad et al., (2009) have found that an agar medium named CHROMagar Vibrio (CV) agar contains a substrates for the bacterial β-galactosidase is a very useful medium to distinguish *V. parahaemolyticus* from other *Vibrio* species by producing a purple colour on this medium due to the reaction of β-galactosidase in this bacterium with the medium substrate. Despite the development of a selective medium, the traditional methods for isolation and identification of *V. parahaemolyticus* remain expensive, time consuming and prone to contamination.

Many *Vibrio* species have variable characters, therefore biochemical and nutritional tests are not sufficient to differentiate them (Glenn, 1976). For example, the phenotypic and genotypic characteristics of *V. harveyi* and *V. camphellii* are close and they exhibit very similar characteristics in biochemical characterisation tests (Pace and Chai, 1989). A set of 28 tests are used for an initial identification of *Vibrio* species. The tests are used on isolates that are suspected of being a *Vibrio* species i.e. Gram-negative, oxidase-positive, glucose-fermenting and grow on any *Vibrio* medium (Alsina and Blanch, 1994). Croci et al., (2007) have reported that false positive results were obtained with using biochemical methods for identification of *V. parahaemolyticus*. Furthermore, identification of environmental *Vibrio* species by using the biochemical tests is time consuming, problematic and difficult due to their great diversity.

To identify and differentiate marine bacteria, the API style of biochemical test kit includes 20 reactions and the profiles are given for clinically important bacteria including *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*. Several kits for biochemical tests have been used to identify suspected isolates of *V.*
parahaemolyticus, including API 20E and API NE and the Crystal Enteric/Non-Fermenter ID Kit (BioMetrix). These kits can identify V. parahaemolyticus in 1-2 days (API 20E), 4 hours (API NE and RapID™ NF PLUS) and 18 hours (Crystal E/NF) (Su and Liu, 2007). Characteristics of V. parahaemolyticus are listed in Table 1.2.

Honda et al., (1980) have characterised immunological techniques as reproducible for detection of the haemolysin-producing pathogenic V. parahaemolyticus. Moreover, Honda et al., (1982) have reported immunological methods for identification of the Kanagawa phenomenon in modified traditional selective media, i.e. bromthymol blue (BTB) - teepol agar and arabinose-ammonium sulphate-cholate agar, for V. parahaemolyticus. These methods are helpful to isolate V. parahaemolyticus and identify the Kanagawa phenomenon on the same plate. The immune-halo test was performed by inoculating bacteria onto an agar medium that contained either anti-haemolysin immunoglobulin G (IgG) or anti-haemolysin antiserum (Honda et al., 1980). Honda, et al., (1985) have recorded the development of an enzyme-linked immunosorbent assay (ELISA) to detect the TDH.
Table 1.2 Biochemical characteristics of *V. parahaemolyticus*\(^a\), adapted from (Lam and Yeo, 1980).

<table>
<thead>
<tr>
<th>Positive</th>
<th>Negative</th>
</tr>
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<tbody>
<tr>
<td>Indole</td>
<td>Methyl red.</td>
</tr>
<tr>
<td>Simmons citrate.</td>
<td>Voges-Proskauer.</td>
</tr>
<tr>
<td>Motility</td>
<td>Hydrogen sulfide.</td>
</tr>
<tr>
<td>Oxidation-fermentation.</td>
<td>Arginine dihydrolase.</td>
</tr>
<tr>
<td>Lysine decarboxylase.</td>
<td>Phenylalanine.</td>
</tr>
<tr>
<td>Ornithine decarboxylase.</td>
<td>Malonate.</td>
</tr>
<tr>
<td>Catalase</td>
<td>No acid production from:</td>
</tr>
<tr>
<td>Acid production from:</td>
<td>Lactose, rhamnose, sucrose, adonitol,</td>
</tr>
<tr>
<td></td>
<td>inositol, xylose, sorbitol</td>
</tr>
<tr>
<td>Glucose, maltose, mannitol, arabinose,</td>
<td></td>
</tr>
<tr>
<td>trehalose,</td>
<td></td>
</tr>
<tr>
<td>Growth in 1% peptone water with: 3</td>
<td>No growth in 0% and 10% NaCl</td>
</tr>
<tr>
<td>and 7% NaCl.</td>
<td></td>
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</table>

\(^a\)All tubed media for the biochemical tests were with 3% NaCl.
1.10.2 Molecular identification and detection methods

Proposals for diagnostic techniques involve the use of gene probes. These techniques use oligonucleotide sequences of DNA or RNA, which hybridise to target sequences of the bacterial genome. The use of synthesised oligodeoxyribonucleotide probes labelled with $^{32}\text{P}$ to detect $tdh$ gene in $V. \text{parahaemolyticus}$ has been described by Nishibuchi et al., (1986). Furthermore, Lee et al., (1992) have constructed an oligonucleotide probe labelled with $^{32}\text{P}$ as a method to detect the $tdh$ gene of $V. \text{parahaemolyticus}$ in foods that were contaminated artificially. McCarthy et al., (1999) have developed two non-radioactive probes including digoxigenin (DIG)–labelled and Alkaline Phosphatase (AP)-labelled probes to detect $tlh$ gene in $V. \text{parahaemolyticus}$ to identify $V. \text{parahaemolyticus}$. More recently, Islam et al., (2004) have used such methods for detection of pathogenic $V. \text{parahaemolyticus}$ isolated from the marine environment in Bangladesh which possess $tdh$ gene.

The use of amplification techniques such as polymerase chain reaction (PCR) may permit identification and detection of the bacterium in clinical and environmental samples. Many PCR-based assays have been established to assist in the specific identification of $V. \text{parahaemolyticus}$. DNA gyrase subunit B encoded by the gyrB gene that is responsible for DNA replication has been proposed by Venkateswaran et al., (1998) to distinguish $V. \text{parahaemolyticus}$ from $V. \text{alginolyticus}$. The specific PCR to target gyrB gene to identify $V. \text{parahaemolyticus}$ in shrimp has been developed despite the homology of the gyrB sequences between both these bacteria is being 86.8% (Venkateswaran et al., 1998). However, the gyrB PCR also detected $V. \text{alginolyticus}$ isolates as reported by Kim et al., (1999). The Tox R transmembrane protein encoded by the toxR gene is concerned with the regulation of genes in $Vibrio$ species. A specific PCR to target
the toxR gene to identify *V. parahaemolyticus* has been developed by Kim et al., (1999). Rosec et al., (2009) have reported that the PCR targeting the toxR gene is an efficient and reliable tool for the identification of *V. parahaemolyticus* (Croci et al., 2007). Another specific PCR which targets the *tlh* gene, encoding the thermolabile haemolysin in *V. parahaemolyticus* has been developed by Bej et al., (1999) and Sakata et al., (2012). However, *tlh* was also found in *V. alginolyticus* (Robert-Pillot et al., 2002). Another PCR target is the pR72H fragment, which contains a phosphatidylserine synthetase gene, and non-coding region. The primers are used to amplify an amplicon of either 320 bp or 387 bp in length, that are considered specific for identifying *V. parahaemolyticus* (Robert-Pillot et al., 2002).

Importantly, a multiplex PCR (M-PCR) provides a valuable tool for the quick detection of a strain or species. A M-PCR has for *V. parahaemolyticus* been developed and used to detect *tdh, trh* and *tlh* genes simultaneously in samples from shellfish (Bej et al., 1999, Terzi et al., 2009). Recently, M-PCR has supplied a rapid and sensitive technique to detect specific pathogenic bacteria in marine environments (Kong et al., 2002). A M-PCR has been developed by Espiñeira et al., (2010) to detect simultaneously the five pathogenic *Vibrio* species in seafood samples by M-PCR to target the *dnaJ* gene in a single reaction producing species-specific amplicon 450 bp for *V. parahaemolyticus*, 144 bp for *V. alginolyticus*, 564 bp for toxigenic *V. cholerae O1*, 177 bp for *V. mimicus* and 412 bp for *V. vulnificus*; and applied this PCR to detect directly all these pathogenic species from stool samples (Nhung et al., 2007). Tyagi et al., (2009) have detected pathogenic *V. parahaemolyticus* by a Quantitative PCR (Q-PCR) method that was established to detect *tdh* positive *V. parahaemolyticus* bacteria in oysters and in pure cultures (Blackstone et al., 2003). Moreover, this technique requires less time and appears
to be reliable and accurate (Wittwer et al., 2001, Parveen et al., 2008). A multiplex Q-PCR has been demonstrated to detect \textit{tdh}, \textit{trh}, \textit{tlh}, and ORF8 in the same reaction by using four sets of primers and four probes labelled with four different fluorophores for more rapid detecting of total and virulent forms of \textit{V. parahaemolyticus} in oysters. This assay has applications in the seafood industries to ensure food safety (Ward and Bej, 2006).

More recently, a quick, cheap, highly sensitive and specific nucleic acid amplification technique, named loop-mediated isothermal amplification (LAMP), has been developed using a DNA polymerase which has strand displacement activity, and 4-6 designed primers that specifically recognise the 6-8 regions of the target DNA under isothermal conditions (Notomi et al., 2000). It has been used for detecting many bacteria such as \textit{Salmonella} (Hara-Kudo et al., 2005), \textit{V. vulnificus} (Han and Ge, 2008), and \textit{Porphyromonas gingivalis}, \textit{Treponema denticola} and \textit{Tannerella forsythia} from periodontal infections (Yoshida et al., 2005).

Promising results have been yielded for detection of total \textit{V. parahaemolyticus} in seafood samples using the LAMP method (Yamazaki et al., 2008, Sun et al., 2012). Additionally, Chen and Ge, (2010) have developed a \textit{toxR}-based LAMP method for total detection of \textit{V. parahaemolyticus} in oysters, and reported that \textit{toxR}-based LAMP is more sensitive than \textit{toxR}-based PCR. LAMP is also used for detection of pathogenic \textit{V. parahaemolyticus} targeting \textit{tdh} gene (Nemoto et al., 2009).
1.10.3 Molecular typing techniques

1.10.3.1 Randomly amplified polymorphic DNA analysis (RAPD)

Typing techniques that investigate the entire genome are frequently more discriminatory than techniques that investigate variation at a specific locus. RAPD-PCR is one such technique that is described as a sensitive method for detecting slight differences in genomic structure in different bacteria by employing short oligonucleotide primers of random sequence (Welsh and McClelland, 1990). This method has been successfully used in the analysis of *V. parahaemolyticus* and other *Vibrio* species (Wong and Lin, 2001). This technique distinguishes strains of food borne pathogens such as *V. parahaemolyticus* depending on differences in nucleotide sequences in the whole genome. Sudheesh et al., (2002) have used RAPD-PCR for typing and differentiating between *V. parahaemolyticus* and *V. alginolyticus* strains isolated from cultured shrimps. Bilung, et al., (2005a) have examined the genetic relatedness among the *V. parahaemolyticus* strains by RAPD-PCR. It was revealed to be a robust, quick and sensitive typing technique to discriminate *V. parahaemolyticus* strains. Najiah et al., (2003) have demonstrated that no correlation exists between genetic and geographic origins of *V. parahaemolyticus* isolates from diseased fish and shrimp in brackish water ponds in Malaysia. In addition, in Taiwan, Wong, et al., (1999) described that geographical distances of clinical *V. parahaemolyticus* strains involved in gastroenteritis outbreaks did not have any important effect on the group of the RAPD types. Ultimately, the important advantages of RAPD-PCR are (i) its applicability to problems where limited quantities of DNA are available (ii) its suitability for working on anonymous (unknown) genomes and (iii) its low expense and good efficiency (Hadrys et al., 1992).
1.10.3.2 Pulse field gel electrophoresis (PFGE)

Pulse field gel electrophoresis (PFGE) for *V. parahaemolyticus* was first reported in 1996 (Lu et al., 2000). PFGE is used to distinguish isolates of the same species of pathogenic bacteria (Bohm and Karch, 1992), including numerous *Vibrio* species, e.g. *cholerae* (Mahalingam et al., 1994). It has been used relatively little in environmental research, however, Tamplin et al., (1996) have used it extensively in epidemiological studies. This technique depends on the migration of large restriction endonuclease DNA fragments in gels. This migration occurs after cleavage of genome DNA with site-specific and low frequency cutting restriction endonucleases (Bohm and Karch, 1992). In recent years, PFGE has been used to detect and identify *V. parahaemolyticus* (Wong et al., 2000). It has high discrimination, reproducibility and is useful for typing pathogenic strains of *V. parahaemolyticus* (Liu et al., 2009). Furthermore, Ellingsen et al., (2008) have used PFGE to examine Norwegian *V. parahaemolyticus* isolated from the environment including that carry *trh* gene, and compare them to clinical isolates, the authors did not find any evidence that these isolates have contributed to disease and there were no obvious similarities between clinical and environmental of *V. parahaemolyticus* isolates tested. Wagley et al., (2008) have reported that PFGE showed a high degree of genetic diversity in *V. parahaemolyticus* isolates and it was not found relationship between isolates that isolated from similar seafood or origins. However, Miah (2009) have used PFGE for typing of *V. parahaemolyticus* and found difficulties such as a lack of typing in many samples of DNA of *V. parahaemolyticus*.

1.10.3.3 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) uses a restriction enzyme to cut purified genomic DNA at specific sites. Electrophoresis is used to separate the
DNA fragments generated (Linton et al., 1995). This technique is a valuable tool for typing strains of some species of bacteria including *V. cholerae* (Dalsgaard et al., 1995). The RFLP-PCR has been used to amplify the 16S rRNA and subsequent restriction of the PCR products with endonuclease to create banding patterns when separated on agarose gel (Chun et al., 1999). In addition, this method has been used to characterise genes encoding components of polar flagella in *V. parahaemolyticus* (Marshall et al., 1999). Figure 1.1 shows the procedural steps of molecular typing methods.

<table>
<thead>
<tr>
<th>RAPD</th>
<th>PFGE</th>
<th>RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Amplification</td>
<td>Embed organisms</td>
<td>PCR Amplification</td>
</tr>
<tr>
<td>↓</td>
<td>in agarose plug</td>
<td>↓</td>
</tr>
<tr>
<td>Gel Electrophoresis</td>
<td>↓</td>
<td>Restriction</td>
</tr>
<tr>
<td>↓</td>
<td>Protease digestion</td>
<td>Endonuclease digestion</td>
</tr>
<tr>
<td>Gel Staining</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>↓</td>
<td>Restriction</td>
<td>Gel Electrophoresis</td>
</tr>
<tr>
<td>Interpretation</td>
<td>Endonuclease digestion</td>
<td>↓</td>
</tr>
<tr>
<td>↓</td>
<td>Gel Staining</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Electrophoresis</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>Interpretation</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 1.1 The technical stages for three molecular typing methods.*

Modified from Olive and Bean, (1999).
1.10.3.4 Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

The ERIC sequence was first defined in *E. coli*, and is found in *Salmonella typhimurium* and other species including *Klebsiella pneumonia* and *V. cholerae* (Hulton et al., 1991). ERIC sequences are 126 bp long and seem to be restricted to transcribed genomic regions, either in un-translated regions upstream or downstream of open reading frames or in the intergenic regions of operons of polycistrons (Hulton et al., 1991). ERIC-PCR is helpful for assessing epidemiological and genetic relationships among *V. parahaemolyticus* isolates (Marshall et al., 1999). Bhowmick et al., (2008) used ERIC-PCR as a rapid method for molecular epidemiological typing of *V. parahaemolyticus* because it has a potential discriminating ability. Khan et al., (2002) have used ERIC-PCR to find a unique sequence band at 850 bp and using this sequence they developed a rapid PCR method to precisely classify O3:K6 isolates of *V. parahaemolyticus* that were isolated from a United States outbreak.

1.10.3.5 Repetitive extragenic palindromic PCR (REP-PCR).

REP elements were first described in *E. coli* as 35 bp sequences composed of a high conserved inverted repeat with the potential of forming a stem-loop structure (Stern et al., 1984). Moreover, Aranda-Olmedo, et al., (2002) have found this element in *Pseudomonas putida* repeated with a high degree of sequence conservation. Both REP-PCR and ERIC-PCR have been applied to study the genetic variation among non-O1 and non-O139 *V. cholerae* at higher sensitivity levels (Rao and Surendran, 2010). Moreover, Brocchi et al., (2006) have used REP-PCR to differentiate between pathogenic and non-pathogenic of *E. coli* strains. The similarities and differences between ERIC and REP are summarised in Table 1.3.
Table 1.3 The similarities and differences between ERIC and REP\(^1\).

<table>
<thead>
<tr>
<th>The similarities</th>
<th>The differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Their chromosomal locations differ between species.</td>
<td>1- ERIC sequences are much longer (126 bp and REP is 33 bp).</td>
</tr>
<tr>
<td>2- Both are located in non-coding transcribed regions of the chromosome.</td>
<td>2- The estimated number of REP sequences per chromosome is at least 10 times than that of ERIC.</td>
</tr>
<tr>
<td>3- Both include a potential stem-loop structure.</td>
<td>3- Multiple REP sequences are often found at a single chromosomal location whereas all the ERIC identified so far occur singly.</td>
</tr>
<tr>
<td>4- Both can occur in either orientation with respect to transcription.</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Data from Hulton et al., (1991).

1.10.3.6 Multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST)

MLEE has also been reported as a molecular typing method to study the genetic relationship between isolates the same species (Vieira et al., 1998). While, Maiden et al., (1998) have established MLST as an enhanced variation of MLEE. It has been promoted as the most reliable molecular tool for epidemiological studies. MLEE compares the electrophoretic mobility of genes coding the enzyme, whereas MLST allocates alleles directly from the nucleotide sequences (Walk et al., 2007). Vieira et al., (2001) have used the MLEE method to provide an accurate molecular method for distinguishing between \textit{V. cholerae} and \textit{V. mimicus} isolates that are especially helpful for identifying of atypical forms. MLST has been applied to study the differentiation between the pandemic and non-pandemic strains of \textit{V.
cholerae (Kotetishvili et al., 2003). MLST has also been applied to *Salmonella* (Kotetishvili et al., 2002), and *V. parahaemolyticus* (González-Escalona et al., 2008). Allelic variations in the nucleotide sequences of several loci have been observed to discriminate strains by using MLST. Interestingly, diverse typing methods are frequently used in different laboratories for the same pathogens, thus the data are difficult to compare between laboratories and are usually inconvenient for population genetics, evolutionary or phylogenetic studies. In addition, MLEE is used for typing and population genetic analyses of parasites and fungal pathogens (Maiden et al., 1998).

### 1.11 High throughput sequencing

A new DNA sequencing method, high throughput (or ‘next generation’) sequencing technique is faster and more cost effective to allow quick sequencing of whole genomes. The sequence reaction contains the template (single stranded DNA), primer, DNA polymerase, and the enzymes sulfurylase and luciferase. One of the DNA synthesis products and the DNA chain itself is pyrophosphate which is released every time a nucleotide is added to a growing DNA chain. Addition of a nucleotide to the growing DNA chain is signaled by release of pyrophosphate (Lodge et al., 2007).

In terms of high throughput sequencing application, it can be applied to bacteria isolated in pure culture to provide high resolution diagnostic and epidemiological information. Furthermore, high throughput sequencing can be also applied in pathogen biology to screen transposon libraries to identify genes and pathogens that contribute to fitness in different environments (Van Opijnen et al., 2009, Langridge et al., 2009, Goodman et al., 2009, Gawronski et al., 2009).
Azhikina et al., (2010) have used this method to obtain whole pathogen transcriptomes from infected host tissues. It has been also used for RNA sequencing to assess gene expression profiles (Han et al., 2007, Galindo et al., 2009). Simen et al., (2009) have applied high throughput sequencing to the detection of resistance in viral pathogens, such as human immunodeficiency virus.

In recent years, development of next generation sequencing (NGS) has been applied to whole genome sequencing and enabled the rapid construction of many complete microbial genomes. Hu et al., (2011) have used NGS for pathogen comparative genomics and comparing pathogens to less, or non-pathogenic near neighbors. Genome sequence differences between pathogens and non-pathogens have been previously studied but did not give which sequence may be responsible for a specific phenotype, for example, small genomic differences between closely related species of the same genus such as between *Yersinia pestis* and *Yersinia pseudotuberculosis* (Chain et al., 2004), and large genomic differences such as genomic islands within a single species of *Escherichia coli* (Perna et al., 2001). However, using NGS provides more investigation of human pathogens and their mechanisms of virulence and can be applied to pathogen genome comparison and determine pathogenic phenotypes in bacteria.
1.12 Aim and objectives

The overall aim of this work was to develop reliable methods for distinguishing pathogenic and non-pathogenic isolates of *Vibrio parahaemolyticus*.

Project Objectives

i) Collect and check *Vibrio parahaemolyticus* isolates. Confirm identification using the following tests:

   (a) Gram stain, biochemical & nutritional tests.
   
   (b) PCR to identify *V. parahaemolyticus* isolates.

ii) Test clinical and environmental *Vibrio parahaemolyticus* isolates for differences in enzyme activities.

iii) Look for carriage of plasmids and bacteriophages which might carry genes for virulence factors. (a) Use agarose gel electrophoresis to check for the presence of plasmids. (b) Take culture supernatant from different isolates and assay for phage.

iv) Analysis of whole cell proteins and extracellular proteins by SDS-PAGE to look for differences between virulent and avirulent isolates.

v) Test cytotoxic effects of extracellular products of *V. parahaemolyticus* on Caco-2 and CHO-K1 cell lines with view to producing a measure of virulence.

vi) Clone regions of DNA unique to clinical (pathogenic) isolates of *Vibrio parahaemolyticus*, including DNA bands of interest identified in molecular typing methods, and sequence the gene(s) of interest in order to develop PCR detection methods.

vii) Use identified gene sequences as a molecular tool to identify *Vibrio parahaemolyticus* and distinguish virulent and avirulent forms through the development of a multiplex-PCR.
CHAPTER 2

GENERAL MATERIALS AND METHODS
Chapter 2: General Materials and Methods

2.1 Chemicals and reagents

Unless stated otherwise all general laboratory chemicals were obtained from Amersham Biosciences, Bio-Rad Laboratory, Invitrogen, Thermo-Fisher Scientific, or Sigma Aldrich and if possible, were of molecular biology grade. All bacteriological culture media were purchased from Oxoid. PCR related reagents were obtained from Roche Biosciences. Formation and preparation of laboratory reagents, buffers and growth media and the suppliers’ details are shown in Appendix 1. Details of the use of specific reagents can also be found in the Materials and Methods section of individual chapters.

2.2 Bacteriology

2.2.1 Isolates of V. parahaemolyticus and growth media

V. parahaemolyticus isolates are listed in Table 2.1. All isolates were maintained on slants of Marine Salts Agar (MSA) with a final salt concentration of 2 % (w/v), at room temperature (21°C) in the dark. Broth cultures were grown at 37 °C in Tryptone Soya Broth (TSB) supplemented with NaCl 3% (w/v), shaking in a water bath (Clifton NE5-28D) at 70 rpm. A selection of biochemical tests and growth characteristics on several bacteriological media were performed regularly to confirm identity and purity of the cultures.

2.2.2 Additional bacterial species and growth media

Other species and non-Vibrio bacteria are listed in Table 2.2. All halophilic Vibrio species were grown in TSB and MSA supplemented with the appropriate concentration of NaCl. Aeromonas hydrophila and A. salmonicida were grown TSB and TSA at room temperature (21 °C) for two days. Non-Vibrio bacteria were grown in Luria-Bertani (LB) broth overnight at 37 °C.
Table 2.1 *Vibrio parahaemolyticus* isolates used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>CEFAS reference</th>
<th>Obtained from</th>
<th>Location</th>
<th>Reference Strain/Clinical/Environmental</th>
<th>PCR&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
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<td>R1</td>
<td>V05/001</td>
<td>PHLS</td>
<td>PHLS, UK</td>
<td>Clinical, NCTC Reference Strain</td>
<td>-</td>
</tr>
<tr>
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<td>V05/009</td>
<td>NSVS</td>
<td>Oslo, Norway</td>
<td>Clinical</td>
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</tr>
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<td>NSVS</td>
<td>Oslo, Norway</td>
<td>Clinical</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
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<td>NSVS</td>
<td>Oslo, Norway</td>
<td>Clinical</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
</tr>
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<td>C5</td>
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<td>+</td>
</tr>
<tr>
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<td>+</td>
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<td>+</td>
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<td>-</td>
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<tr>
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<td>+</td>
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</tbody>
</table>

<sup>1</sup>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. <sup>2</sup>PCR data for virulence *tdh* and *trh* genes were provided (see chapter 4). Source of environmental isolates (where known) from following species: G: *Crassostrea gigas* (Pacific oyster), M: *Pecten maximus* (Great scallop), S: *Maja squinado* (Common spider crab), E: *Mytilus edulis* (blue mussel).
Table 2.2 *Vibrio* species non-*Vibrio* bacteria strains that used in this study

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Bacterial Strain</th>
<th>Obtained from</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td><em>V. cholerae</em></td>
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<td>Reference strain</td>
</tr>
<tr>
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<td><em>V. mimicus</em></td>
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<td>Reference strain</td>
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<td>Reference strain</td>
</tr>
<tr>
<td>VV2</td>
<td><em>V. vulnificus</em></td>
<td>CEFAS</td>
<td>Environmental</td>
</tr>
<tr>
<td>VV3</td>
<td><em>V. vulnificus</em></td>
<td>CEFAS</td>
<td>Clinical, Japan</td>
</tr>
<tr>
<td>VV4</td>
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<td>CEFAS</td>
<td>Clinical, Israel</td>
</tr>
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</tr>
<tr>
<td>VV6</td>
<td><em>V. vulnificus</em></td>
<td>CEFAS</td>
<td>Clinical, USA</td>
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<tr>
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<td>CEFAS</td>
<td>Clinical isolate</td>
</tr>
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<td>VA2</td>
<td><em>V. alginolyticus</em></td>
<td>CEFAS</td>
<td>Clinical, Japan</td>
</tr>
<tr>
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<td><em>V. alginolyticus</em></td>
<td>FDL²</td>
<td>Fish</td>
</tr>
<tr>
<td>VA4</td>
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<td><em>V. alginolyticus</em></td>
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<td>Fish</td>
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<tr>
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<td><em>V. alginolyticus</em></td>
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<td>Fish</td>
</tr>
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<td>Coral</td>
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<td>UoP</td>
<td>Fish</td>
</tr>
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<td>Fish</td>
</tr>
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<td>NCIMB</td>
<td>Reference strain</td>
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<td><em>Salmonella typhimurium</em></td>
<td>NCIMB</td>
<td>Reference strain</td>
</tr>
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<td>UoP</td>
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<tr>
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<td><em>Klebsiella pneumoniae</em></td>
<td>UoP</td>
<td>N/A</td>
</tr>
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<td><em>Eschericia coli</em></td>
<td>NCIMB</td>
<td>Reference strain</td>
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<td>Ec2</td>
<td><em>E. coli</em></td>
<td>UoP</td>
<td>K12 wild type</td>
</tr>
</tbody>
</table>

¹NCTC National Collection of Type Cultures; ²FDL, Fish diseases laboratory; ³WV, USA; ⁴UoP, University of Plymouth; ⁵NCIMB, National Collections of Industrial Food and Marine Bacteria.
2.3 Extraction of DNA

Genomic DNA was extracted from *V. parahaemolyticus* and other species using “DNeasy® Blood & Tissue Kit” forGram-negative bacteria” following the manufacturer’s instruction.

A 5 ml overnight culture of the organism was placed into a 15 ml Falcon tube, and centrifuged at 2,800 x g for 10 min at 4 C°. Next, the cell pellet was resuspended in 180 µl ATL buffer (cell lysis solution) with gentle pipetting. Samples were mixed with 20 µl proteinase K and vortexed and incubated at 56 °C to lyse the cells until the solution mixture became clear. A 4 µl volume of Rnase ‘A’ solution (100 mg/ml) was mixed with the cell lysate and left for 2 min at room temperature. A 200 µl volume of AL Buffer and 200 µl of ethanol were added and vortexed to yield a homogeneous solution.

Following this, the mixture above was pipetted into a DNeasy Mini spin column and placed in a 2 ml collection tube, centrifuged at 2,400 x g for 2 min and the flow through was discarded. The column was placed in a fresh 2 ml collection tube and 500 µl of AW1 Buffer was added and centrifuged at 2,400 x g for 2 min. The DNeasy Mini spin column was placed in a fresh 2 ml collection tube and 500 µl of AW2 Buffer was added and centrifuged for 4 min at 9,500 x g. To elute DNA, the DNeasy Mini spin column was placed in a fresh microcentrifuge tube and 50 µl molecular grade water was added onto the DNeasy membrane and incubated at room temperature for 1 min and then centrifuged for 1 min at 9,500 x g.
2.4 DNA quantification

The quantity and quality of genomic DNA was established by agarose gel electrophoresis (section 2.9). The genomic DNA was visualised and images of each gel were captured after electrophoresis with a Gel Documentation system (Uvi-Tech, UK) (Appendix I), and the DNA concentration was determined for each isolate by comparison with a range of the following known amounts of standard bacteriophage λ: 0.552 µg, 0.276 µg, 0.138 µg using 'U.V. Photo' software (UVi-Tech). Genomic DNA was also quantified with a UV spectrophotometer using 2 µl volumes (Nano-drop 100 spectrophotometer Lab-Tech, UK).

2.5 PCR primers design

Unless stated otherwise, primers for PCR reactions were designed by using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), a lack of primer-dimer formation was assessed using DNA calculator (Sigma-Genosys). Primer sequences were then run through BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were purchased from MWG Biotech Ltd., Germany.

2.6 Polymerase chain reaction (PCR)

PCR reactions were prepared within a PCR cabinet (Heto-Holten, Denmark) and performed with a DNA thermal cycler MWG Primus 96 Plus (MWG Biotech). Each reaction contained 1x PCR reaction buffer (Roche Applied Science, UK) (Appendix I), 50 pmol of each primer, each deoxynucleoside triphosphate (dNTP) at a concentration of 0.2 mM, 1U of Taq DNA polymerase, 20 ng of bacterial genomic DNA, and the mixture was completed to 50 µl with addition of molecular grade water. To monitor for contamination, a negative control was set up in exactly the same way except that the addition of DNA template was replaced with an
equivalent volume of PCR grade water. Various cycling conditions were used as described elsewhere. Specific details are provided in each individual chapter.

2.7 Extraction of DNA from agarose gels

The Agarose Gel DNA Extraction Kit (Roche Applied Science) was used for extraction of DNA as follows: DNA fragments of interest were separated on an agarose gel using 1x TRIS-borate EDTA buffer (TBE) (Appendix I) running buffer, visualised and isolated by cutting out with a sharp clean and sterile scalpel. These were transferred to pre weighted reaction tubes. A 300 µl of the Agarose Solubilisation Buffer (vial 2) were applied per 100 mg of agarose gel. The Silica Suspension (vial1) was resuspended to obtain a homogeneous suspension, and a 10 µl volume was added, and incubated at 56-60°C for 10 min, with vortexing every 2-3 min. Next, samples were centrifuged for 1 min at 9,500 x g. The pellet was resuspended in 500 µl Nucleic Acid Binding Buffer (vial 3) using a vortex mixer, and centrifuged for 1 min at 9,500 x g. Washing buffer (vial 4) 500 µl was used twice for washing the pellet. Next the tubes were inverted on a tissue to allow pellet to dry at room temperature for 10-16 min. A 25 µl volume of molecular grade water was used to elute DNA and left for 10 min at 56-60° and vortexed every 2-3 min. After centrifugation at 9,500 x g for 1 min the DNA containing solution was transferred to a fresh tube.

2.8 Purification of PCR products

PCR products were purified using the ‘High pure PCR Product Purification Kit’ (Roche Applied Science) following the manufacturers’ instruction. Details of the individual solutions included in this kit can be found in appendix I. The final volume of the reaction mixture of PCR products was adjusted with PCR grade water to 100 µl. A 500 µl volume of Binding Buffer was mixed thoroughly with the PCR
product. The mixture was centrifuged at 9,500 x g for 1 min, on a column (containing the manufacturers filter) and the flow through solution was removed, and the filter washed firstly with 500 µl of Wash Buffer and centrifuged at 9,500 x g for 1 min, and secondly with 200 µl of Wash Buffer and centrifuged at 9,500 x g for 1 min. A 25 µl volume of molecular grade water was added onto the column and placed in a clean 1.5 ml microcentrifuge tube and left at room temperature for 1 min. The DNA was eluted by centrifugation at 9,500 x g for 1 min. This DNA was quantified using the procedure in section 2.4.

2.9 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a TBE buffer. Depending upon the application, it was performed using 0.8%-2% (w/v) agarose made with 1 x TBE buffer. Two sizes of agarose gel were used, 60 ml or 250 ml volume approximately 80 x 100mm or 200 x 200mm slabs. Agarose gels of 60 ml were run for 1 hour at 120V, while 250 ml gels were electrophoresed at 95 V for 5 h using Pharmacia tanks (Biotech, Sweden). Prior to electrophoresis, the samples were loaded with 6x gel loading solution. The sample volumes and concentrations varied according to each analysis and are stated in the Material and Methods section of each chapter. DNA molecular size standards, either 100 bp or 1 kbp (0.3µg) (Invitrogen, UK) (Appendix I) were included on gels to assist in the estimation of the sizes of DNA fragments. Following electrophoresis, the DNA bands were stained by immersing the gel for 20 min in a TBE buffer containing Red gel stain or ethidium bromide (0.5µg/ml), followed by 10 min destaining in distilled water to remove any unbound stain. The stained bands were visualised with UV light (309 nm) using a trans-illuminator and gels were recorded as digital TIFF images using a gel documentation system (UVI-Tech).
2.10 DNA manipulation methods

2.10.1 Ligation of DNA molecules

T4 DNA Ligase was used to ligate DNA fragments of interest to pGEM-T Easy vector (vector). The reaction mixture contained 5µl of 2X Rapid Ligation Buffer, 1µl of vector (50 ng), 3 µl of purified of DNA (insert) and 1µl of T4 DNA Ligase. In control reactions insert DNA provided in the kit was used. The ligation mixture was kept at 4°C overnight.

2.10.2 Transformation of Escherichia coli

E. coli JM109 was used for transformation of ligation products as follows: 2µl of the ligation reaction were added to 50 µl of competent cells and mixed gently. To determine the transformation efficiency of the E. coli JM109, a 0.1 µg sample of uncut plasmid was also used. The tubes were placed on ice for 20 min, next the tubes were incubated at 42 °C for 1 min (heat shocked), and then the tubes returned to ice for 2 min. A 950 µl volume of SOC medium (Appendix I) was added to the tubes containing cells transformed with ligation reaction and 900 µl to the tube containing cells transformed with uncut plasmid and incubated for 1.5 hour at 37°C with shaking 150 rpm.

2.10.3 Blue/white screening for recombinant clones

A 50 µl and 100 µl samples of each transformation culture of transformants were spread onto duplicate LB/Ampicillin/X-Gal/IPTG plates (Appendix I). Plates were incubated at 37 °C for 24 - 48 h, and examined for the presence of blue (lac⁺, non-recombinant), and white (lac⁻, recombinant) colonies. Following the transformation of E. coli JM109 with ligated DNA the recombinants (white colonies) was selected.
2.10.4 Purification of plasmid DNA

Recombinant plasmid DNA was isolated from *E. coli* using the Miniprep plasmid DNA extraction kit (GenElute™ Plasmid Miniprep Kit) (Sigma, UK) as follows: Recombinant *E. coli* cultures were grown in LB medium overnight at 37 °C. A 5 ml volume of recombinant *E. coli* culture was centrifuged and 200 µl of the resuspension solution was used to resuspend the bacterial pellet until homogenous. A 200 µl volume of the lysis solution was added. The contents were mixed by gentle inversion (6-8 times) without vortexing. A 350 µl volume of the Neutralisation/Binding Solution was added. Tubes were inverted about 6 times. The cell debris was pelleted by centrifuged at 9,500 x g for 10 min and the supernatant was recentrifuged to give clear lysates. Next, a column of GenEluteMiniprep Binding was inserted into a provided microcentrifuge tube to prepared by adding 500 µl volume of the Column Preparation Solution, and centrifuged at 9,500 x g for 1 min. Afterwards clear lysates were transferred to the columns and centrifuged at 9,500 x g for 1 min and the flow-through liquid was discarded. A 500 µl volume of the Optional Wash Solution was added and centrifuged at 9,500 x g for 1 min and the flow-through liquid discarded. A 750 µl volume of Wash Solution was added and centrifuged at 9,500 x g for 1 min to remove residual salt and other contaminants, and then recentrifuged to remove ethanol. The column was transferred to a clean tube and 50 µl of molecular biology reagent water was added to the column. The eluted plasmid DNA was recovered by centrifugation at 9,500 x g for 1 min.

2.10.5 Verifying cloning by using restriction enzyme analysis

The cloning was verified by digestion with restriction enzymes. Restriction digestion with *EcoR1* was performed in a volume of 20µl containing 100 ng of plasmid DNA, 2µl of restriction endonuclease buffer, 0.2µl of Acetylated BSA,
10µg/µl, 1µl of *EcoR1* (10µ/µl) and to complete the final volume with analytical grade H₂O to 20µl. The digest was incubated overnight at 37 °C to ensure complete digestion. Agarose gel electrophoresis was used to analyse the products of endonuclease digestion (section 2.9).

2.11 DNA sequencing and sequence analysis

The cloned DNA was sequenced by MWG-Biotech Ltd., Germany. Computer analysis of the DNA sequence data was performed via the National Centre for Biotechnology Information (NCBI) using GenBank databases search and BLAST programs (NCBI) and the nucleotide sequences were submitted to GenBank.
CHAPTER 3

IDENTIFICATION AND CONFIRMATION OF *VIBRIO PARAHAEOMOLYTICUS* ISOLATES
Chapter 3: Identification and Confirmation of *Vibrio parahaemolyticus* Isolates

3.1 Introduction

There are many techniques used to identify *V. parahaemolyticus*. The selective medium thiosulfate/citrate/bile salt/sucrose agar (TCBS) has been extensively used for the identification of *V. parahaemolyticus* by producing blue green colonies because this organism does not ferment sucrose. CHROMagar™ *Vibrio* (CV) medium is also used to identify and confirm *V. parahaemolyticus* by revealing purple colonies using a chromogenic substrate (Hara-Kudo et al., 2003).

O'hara et al., (2003) have reported that *Vibrio* species that were isolated from patients can be traditionally identified by commercial identification systems and biochemical tests, but may not always be accurate. Moreover, the biochemical tests are not sufficient or accurate enough to identify all *V. parahaemolyticus* isolates (Robert-Pillot et al., 2002). Furthermore, Takahashi et al., (2005) have reported that traditional typing methods such as conventional culture methods and biochemical profiles have failed to distinguish between virulent and avirulent strains of *V. parahaemolyticus* because there are no clear growth features for virulent strains that could be distinguished from avirulent strains. Nowadays, a specific PCR can be used to confirm the identification of *V. parahaemolyticus*.

A number of species specific PCR tests are available to identify the presence of specific genes in *V. parahaemolyticus*. Gene *thl* encoding thermolabile haemolysin is present in virulent and avirulent strains of *V. parahaemolyticus* but this haemolysin does not contribute to the pathogenicity of this organism and has led workers to develop a PCR using this gene to identify *V. parahaemolyticus* (Taniguchi et al., 1986, Bej et al., 1999). Moreover, a specific PCR targeting
pR72H fragment was also developed for detection *V. parahaemolyticus* in shellfish (Lee et al., 1995a, Cordova et al., 2002). A specific PCR which detects *toxR* gene that encodes the Tox R transmembrane protein have been developed for detection *V. parahaemolyticus*, however, *tlh* is also found among *V. alginolyticus* (Xie et al., 2005). Moreover, a specific PCR to detect the *gyrB* gene for identifying *V. parahaemolyticus* has been developed by Venkateswaran et al., (1998). Recently, Croci et al., (2007) have compared PCR for detection of *toxR* gene, *tlh* gene, and the pR72H fragment for identification of *V. parahaemolyticus*. They found the PCR that detected *toxR* gene gave the optimal results for concordance, inclusivity and exclusivity as confirmed by Rosec et al., (2009).

A collection of *V. parahaemolyticus* isolations was obtained from 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, as previously described (A. Miah, PhD thesis, University of Plymouth).

Before proceeding with the present study, it was necessary to assess phenotypic characters amongst clinical and environmental isolates of this organism, and to confirm the identity of *V. parahaemolyticus* isolates by selective media, standard biochemical tests including Gram staining, oxidase, catalase, motility, sucrose and lactose fermentative, and confirm by specific PCR targeting *toxR* gene.
3.2 Materials and methods

3.2.1 Subculture and identification of *V. parahaemolyticus* on selective media and biochemical test

All bacterial isolates were recovered for use from marine salts agar (MSA) slopes and cultured overnight at 37°C in appropriate volumes of marine salt broth (MSB) shaking at 70 rpm or Tryptone Soy Broth (TSB) with a final NaCl concentration of 3 % (w/v). Bacterial isolates were streaked on thiosulphate citrate bile sucrose agar (TCBS) and HiCrome™ Vibrio agar plates and incubated for 24 hours at 37°C. The pure colonies were streaked onto fresh HiCrome™ Vibrio agar for purity. Stained slide smears from pure cultures were examined under the microscope for their Gram reaction. The following biochemical tests (oxidase, catalase, motility, sucrose and lactose fermentative) were also performed as further characters to identify the isolates.

3.2.2 Confirmation by PCR assay

All *V. parahaemolyticus* isolates were tested by specific PCR for the toxR gene to confirm identity as *V. parahaemolyticus*. DNA was extracted from all the isolates as described in section 2.3 to use it as a template for PCR. Specific primers *ToxR-4* (F: 5'-GTCTTCTGACGCAATCGTTG -3') and *ToxR-7* (5'-R: ATACGAGTGGTTGCTGTCATG -3') to target regulatory gene *ToxR* (Kim et al., 1999) were used to perform a specific PCR for identification *V. parahaemolyticus* isolates. The PCR reaction was performed as described in section 2.6. The PCR cycling condition was 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 59 °C and 2 min at 70 °C followed by a final extension at 70 °C for 5 min. The PCR products were electrophoresed as described in section 2.9. For confirmatory purposes, the PCR products produced by selected isolates were sequenced by
MWG Biotech. These sequences, together with the nucleotide sequences of *V. parahaemolyticus* strain RIMD 2210086 ToxR gene were aligned using the online programs using software Multalin http://www-archbac.u-psud.fr/genomics/multalin.html (Corpet, 1988). GenBank database was used to compare the sequences with those from other organisms.
3.3 Results

3.3.1 Subculture on selective media and biochemical tests.

Isolates were previously identified and confirmed as *V. parahaemolyticus* by a previous study in this school (Miah, 2009) and by colleagues at CEFAS. The results here confirmed the identity of isolates before using in the current study.

All 55 strains of *V. parahaemolyticus* formed green colonies on TCBS (Figure 3.1 and Table 3.1). *V. mimicus* and *V. vulnificus* are also formed green colonies (Table 3.2). TCBS cannot differentiate *V. parahaemolyticus* from *V. mimicus* and *V. vulnificus*. While, *V. cholerae* and *V. alginolyticus* isolates exhibit distinct yellow colonies upon acid production with sucrose fermentation which alters the pH and is indicated by the dyes bromothymol blue and thymol blue. As illustrated in Figure 3.1 and Table 3.1, *V. parahaemolyticus* formed green or blue green colour on HiCrom™ *Vibrio* agar. Therefore, depending on the formation of blue green colonies on this medium, it could differentiate *V. parahaemolyticus* from *V. mimicus* and *V. vulnificus*. In terms of biochemical tests, all *V. parahaemolyticus* isolates were Gram-negative curved rods, catalase and oxidase positive and motile (Table 3.1).

3.3.2 PCR

PCR was performed to confirm phenotypic identification of *V. parahaemolyticus* isolates. The specific PCR targeted the *toxR* gene to produce a 368 bp amplicon which was found in all *V. parahaemolyticus* isolates tested (Figure 3.2 and Table 3.1). No band was seen with any of the other *Vibrio* and non-*Vibrio* species tested (Figure 3.2 and Table 3.2). The nucleotide sequence analysis confirmed the identity of selected PCR products (Figure 3.3).
Table 3.1 Biochemical and nutritional tests of *Vibrio parahaemolyticus* isolates that used in this study

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Bacterial Strain</th>
<th>Colour of colony</th>
<th>Biochemical tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TCBS medium</td>
<td>HiCrom Vibrio agar</td>
</tr>
<tr>
<td>R1</td>
<td>Clinical, NCIMB Reference</td>
<td>Green</td>
<td>Blue green</td>
</tr>
<tr>
<td>C1</td>
<td>Clinical, Norway</td>
<td>Green</td>
<td>Blue green</td>
</tr>
<tr>
<td>C2</td>
<td>Clinical, Norway</td>
<td>Green</td>
<td>Blue green</td>
</tr>
<tr>
<td>C3</td>
<td>Clinical, Norway</td>
<td>Green</td>
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<tr>
<td>C4</td>
<td>Clinical, Norway</td>
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</tr>
<tr>
<td>C5</td>
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+, positive; -, negative
Table 3.2 Biochemical and nutritional tests of *Vibrio* species and non-*Vibrio* bacteria strains that used in this study

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+, positive; -, negative; ND; Not determined
Figure 3.1 Appearance of *Vibrio parahaemolyticus* (a) on TCBS agar medium (b) on HiCrom™ *Vibrio* agar medium.
Figure 3.2 Agarose gel electrophoresis of PCR amplified products using specific primers to detect toxR gene in V. paraahaemolyticus.
Lanes M, 100-bp molecular mass marker. (a) Lane 1, negative control; Lanes 2-12 clinical V. paraahaemolyticus isolates; Lane 13, NCTC Reference strain; Lanes 14-28 environmental V. paraahaemolyticus. (b) Lanes 1-11 clinical; Lanes (12-27) environmental V. paraahaemolyticus Lane 28, negative control. (c) Lane 1, NCIMB Reference strain (R1); Lanes 2-23 Vibrio species, lanes 23-27 non-Vibrio bacteria including (A. hydrophila, A. salmonicida, P. fluorescence, S. typhimurium, Pro. vulgaris, K. pneumoniae, E. coli); lane 28, negative control.
### Figure 3.3 Alignment of the toxR sequence in isolates of *V. parahaemolyticus*. RIMD 2210086 is a sequenced *V. parahaemolyticus* strain

ToxR gene under accession number AY527397.1.

Sequences C10 and E6 from clinical and environmental isolates respectively and R1 is NCTC reference strain. Consensus regions are shown in red and denoted by asterisk; black and blue show deletions or substitutions.
3.4 Discussion

All *V. parahaemolyticus* isolates formed green colonies on TCBS. However, *V. mimicus* and *V. vulnificus* also formed green colonies, that leads to problems to distinguish them from *V. parahaemolyticus*, consequently TCBS may not differentiate *V. parahaemolyticus* from other enteropathogenic vibrios (Duan and Su, 2005b). Moreover, a number of researchers reported that using this medium lead to several limitations and difficulties for the isolation of this bacterium for example, Miwa et al., (2003) have used MPN-TCBS method for isolation and enumeration of *V. parahaemolyticus* from seafood samples that were spiked and naturally contaminated with this bacterium, authors have found difficulties because many colonies other than *V. parahaemolyticus* such as those of *V. alginolyticus* have overgrown on TCBS medium with this method, Miwa et al., (2003) have also used a MPN-PCR method for detection and enumeration of *V. parahaemolyticus* in seafood. The authors found that MPN-PCR more reliable than the MPN-TCBS method.

A new chromogenic medium was developed by Hara-Kudo et al., (2001) for identification *V. parahaemolyticus* and specifically differentiate this bacterium from other bacteria (Blanco-Abad et al., 2009). Furthermore, Eddabra et al. (2011) have reported that the specificity of chromID Vibrio medium for *V. parahaemolyticus* was more than TCBS medium.

In order to enhance the identification of *V. parahaemolyticus* (compare with TCBS medium) HiCrom™ Vibrio agar was used to distinguish *V. parahaemolyticus* from other bacteria based on the reaction between the chromogenic substrate in the medium and β-galactosidase in bacteria rather than sucrose fermentation in TCBS agar (Hara-Kudo et al., 2001). The results of current study indicated that HiCrom™
Vibrio agar could be used for identification of *V. parahaemolyticus* because it could distinguish *V. parahaemolyticus* from *V. mimicus* and *V. vulnificus* depending on the formation of blue green colonies on this medium, while *V. mimicus* gave creamy colonies and *V. vulnificus* gave pale green colonies on HiCrom™ Vibrio agar. While using TCBS did not differentiate *V. parahaemolyticus* from *V. mimicus* and *V. vulnificus* because all these three species do not ferment sucrose and therefore gave green colonies. Recently, Eddabra et al., (2011) have reported that isolation of *V. parahaemolyticus* from stool samples was 100% accurate by chromID Vibrio medium, while only 93% by TCBS medium.

A limited number of methods were used to study the biochemical characteristics of *V. parahaemolyticus* to assess any differences between clinical and environmental isolates. Initial confirmatory biochemical test results were reliable with the typical features of *V. parahaemolyticus* including (Gram negative, motile, oxidase and catalase positive, sucrose and lactose negative). These six biochemical tests were performed with associated with selective media and species specific PCR targeted toxR gene. Sucrose fermentation was used to differentiate *V. parahaemolyticus* from *V. alginolyticus*, *V. cholerae* and *V. harveyi*. Lactose fermentation was used to differentiate *V. parahaemolyticus* from *V. vulnificus*. Gram stain, catalase, oxidase and motility were traditionally used to check the isolates.

However, difficulties are found with the identification of *V. parahaemolyticus* strains isolated from foodstuffs using biochemical tests because the samples may contain related Vibrio species or yet uncharacterised bacteria that may give similar results (Thompson et al., 2006).
The growth characteristics that obtained in the present study did not distinguish between clinical and environmental isolates. Kelly and Stroh, (1989) have demonstrated that conventional tests of the clinical and environmental isolates of *V. parahaemolyticus* showed similar reaction. Furthermore, during an outbreak of *V. parahaemolyticus* food poisoning in Madrid and Barcelona, there were similar biochemical reactions amongst clinical and environmental isolates from marine environments near the outbreak place (Lozano-León et al., 2003). Furthermore, Robert-Pillot et al., (2002) have reported that using biochemical tests to identify clinical *V. parahaemolyticus* isolates were direct and accurate, while, with environmental isolates is difficult to differentiate them correctly from other *Vibrio* species, particularly *V. alginolyticus* because the phenotypic inconsistency of environmental isolates. Robert-Pillot et al., (2002) have used Api 20E to differentiate between *V. parahaemolyticus* and *V. alginolyticus* by sucrose fermentation, however, most *V. parahaemolyticus* and few strains of *V. alginolyticus* cannot ferment sucrose, therefore, sucrose fermentation is not enough and may result in misidentification of these strains (Alsina and Blanch, 1994). The biochemical and nutritional tests have drawbacks that they are time and labour consuming (i.e., isolation, purification and incubation of reactions require 2 to 6 days), not reliable, expensive, may lead to false negative or false positive results, and that the analysis of results needs particular training (Tarr et al., 2007). Therefore, nowadays workers use molecular techniques to overcome the limitation of phenotypic techniques and any ambiguous identification. Identification *V. parahaemolyticus* isolates by using a PCR method to detect the specific *toxR* gene (368 bp PCR product) was more reliable and quicker than biochemical tests, and successfully amplified from all *V. parahaemolyticus* isolates, these results supported that this gene is used to identify *V.
parahaemolyticus isolates (Kim et al., 1999, Sujeewa et al., 2009, Zulkifli et al., 2009). Sequencing of selected PCR products and sequence analysis for homology was performed to verify the identity of PCR products and confirm the results.

A PCR targeting toxR gene is a simple, quick and specific technique; it can identify the isolates in one day and it was used in this study because it is highly specific and reliable (Dileep et al., 2003). Therefore, PCR targeting toxR gene was successfully used in current study to confirm all isolates of V. parahaemolyticus.
CHAPTER 4

SURVEYING VIBRIO PARAHAEOMOLYTICUS ISOLATES FOR PUTATIVE VIRULENCE FACTORS

Results from this Chapter have been presented at SfAM 2010 Summer Conference, Applied microbiology, Brighton, 2010, UK, and Vibrio 2010, Biloxi, Mississippi, USA, 2010 and Marine Institute Conference 2010, Plymouth University, 2010.
Chapter 4: Surveying *Vibrio parahaemolyticus* Isolates for Putative Virulence Factors

4.1 Introduction

Many human pathogenic species of the genus *Vibrio* produce various virulence factors; for example, haemolysin is an exotoxin that lyses erythrocyte membranes with the liberation of haemoglobin. There are two types of haemolysis. The complete degradation of haemoglobin is called β-haemolysis and the incomplete degradation of haemoglobin is known as α-haemolysis (Zhang and Austin, 2005). Wagatsuma has classified *V. parahaemolyticus* isolates into two types: haemolytic and non-haemolytic on special high salt medium named Wagatsuma agar (Miyamoto et al., 1969). Almost all clinical *V. parahaemolyticus* isolates exhibit the Kanagawa phenomenon (KP) beta-type haemolysis on Wagatsuma agar (Izutsu et al., 2008).

Virulent strains of *V. parahaemolyticus* carry haemolysin genes (*tdh* and/or *trh*). These genes are considered as markers for pathogenicity (Sujeewa et al., 2009). Some other *Vibrio* species also possess the *tdh* gene including *V. mimicus* and *V. hollisae* (Nishibuchi and Kaper, 1995). The presence of KP\(^+\) strains carrying the *tdh* gene in *V. parahaemolyticus* isolated from the environment was reported by Cabrera-Garcia et al., (2004). Thus KP\(^+\) has been used as a clinical indicator for the virulence of *V. parahaemolyticus*. It has been recognised as a marker for discriminating pathogenic from non-pathogenic strains (Miyamoto et al., 1969, Honda and Iida, 1993). However, Izutsu et al., (2008) have reported that strains of human pathogenic *V. parahaemolyticus* include both KP\(^+\) and KP\(^-\) isolates. While haemolysins and proteases have been considered principal virulence factors of pathogenic *V. parahaemolyticus* (Miyamoto et al., 1969, Park and Chang, 2003),
other factors may be of importance. Many urease positive bacteria are pathogenic to humans, and it is considered as a marker of virulence in those bacteria (Jones et al., 1990, Johnson et al., 1993, Mobley et al., 1995). For example, urease is essential for colonisation of the stomach and plays a role in *Helicobacter pylori* pathogenicity (Hawtin et al., 1990, Evans et al., 1991). Suthienkul et al., (1995) have reported that the urease positive phenotype of *V. parahaemolyticus* isolated from patients strongly correlated with the presence of the *trh* gene. But KP- *V. parahaemolyticus* isolates that are positive for urease have been described in several reports (Magalhães et al., 1992, Honda et al., 1992a, Cai and Ni, 1996, Okuda et al., 1997a).

Microbial phospholipases are also known to disrupt host cell membranes and may play a main role in the infection process (Titball, 1993). Interestingly, a connection between haemolysis and phospholipase has been demonstrated in a number of studies of *V. parahaemolyticus* (Shinoda et al., 1991, Lee et al., 2002b, Zhong et al., 2006).

High throughput sequencing based methods have been used to differentiate between pathogenic and non-pathogenic strains, for example, it has been apply to investigate a putative methicillin-resistant *Staphylococcus aureus* (MRSA) outbreak and differentiate between outbreak and non-outbreak isolates (Köser et al., 2012). It has also been used for *Mycobacterium tuberculosis* outbreak investigation (Gardy et al., 2011) and *Acinetobacter baumannii* (Lewis et al., 2010). Furthermore, high throughput sequencing has recently been applied to vibrios such as *V. vulnificus* for comparative analysis of clinical and environmental genotypes (Morrison et al., 2012).
There are a number of studies of the incidence of plasmids within *Vibrio* species (Pedersen and Larsen, 1995, Depaola et al., 2003b, Di Lorenzo et al., 2003, Dunn et al., 2005), particularly pathogenic vibrios. Both plasmids and bacteriophages can encode virulence factors which convey pathogenicity to bacterial hosts (Betley et al., 1986). The cryptic plasmid of *V. cholerae* is frequently associated with filamentous phages; it has been detected in all toxigenic strains of this bacterium and contributes in the biology of CTXφ phages (Rubin et al., 1998). There are some phages that infect *V. parahaemolyticus* such as VpV262 (Hardies et al., 2003). Crothers-Stomps et al., (2010) have isolated lytic phage from *V. harveyi*, and Vidgen et al., (2006) reported that phage VHML of *V. harveyi* causes changes in the phenotypic profile of this bacterium.

Many clinical *Vibrio* species of marine fish have plasmids (Tsai and Yeh, 1993). Pedersen and Larsen, (1995) reported that the virulence plasmid pJM1 has been found in pathogenic strains of *V. anguillarum*, but not in non-pathogenic forms of the bacterium. Furthermore, Di Lorenzo et al., (2003) confirmed that plasmid pJM1 of *V. anguillarum* plays a role in pathogenicity of this organism. Terai et al., (1991) have demonstrated that *tdh* genes can sometimes be found on plasmid DNA, despite most copies of these genes being located on chromosomal DNA. Guerry and Colwell (1977) have suggested that plasmids in *V. parahaemolyticus* may be involved in the production of Kanagawa haemolysis.

Sodium dodecyl sulphate - poly acrylamide gel electrophoresis (SDS-PAGE) is one of the most performed molecular methods to characterise macromolecules of bacteria (Durrani et al., 2008). SDS-PAGE has been used to characterise the soluble whole cell protein profiles of bacteria such as *Corynebacterium* (Jackman
and Pelczynska, 1986) *Lactococcus* species (Elliott et al., 1991) and *Leuconostoc* species (Elliott and Facklam, 1993).

Some pathogenic bacteria produce extracellular products (ECPs) that contain important virulence factors. SDS-PAGE has also been used to examine the ECPs profile and determine the molecular weight of proteins. For example, Takeda et al., (1978) have used SDS-PAGE to determine the molecular weight of TDH in *V. parahaemolyticus* and found it to be composed of two subunits of 21 kDa. Furthermore, Lee et al., (2002a) have purified serine protease (protease A) from ECPs of *V. parahaemolyticus* and its molecular weight was found to be 43 kDa, and it is suggested to play a role in pathogenicity of *V. parahaemolyticus*.

Furthermore, Labreuche et al., (2006) have demonstrated that ECPs harvested from a 2 day old culture of pathogenic *V. aestuarianus* were found to be lethal to oysters, and suggested that ECPs contributed to the pathogenicity of this pathogen.

This chapter uses a number of techniques to look for differences between virulent clinical isolates and mainly avirulent environmental forms. Notably it:

i) Compares clinical and environmental isolates of *Vibrio parahaemolyticus* for activities of a variety of enzymes that may contribute to pathogenicity.

ii) Investigates the presence of plasmid and phage which might carry genes for virulence factors.

iii) Analyses whole cell protein and extracellular products by SDS-PAGE to look for differences between clinical and environmental isolates of *V. parahaemolyticus*. 

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4.2 Material and methods

4.2.1 Kanagawa phenomenon

KP haemolysis was examined as previously described (Wagatsuma, 1968, Takeda, 1983). Briefly, aliquots (10 µl) of overnight cultures were streaked on to Wagatsuma agar medium containing 5% (v/v) fresh rabbit defibrinated blood (or saline suspension of red blood cells) (Appendix I) and incubated at 37 °C for 48 h. The isolates exhibiting clear beta-haemolytic zones were designed as positive for the KP. The haemolysis around the bacterial growth was registered as: ++++, strong (large >3 mm, clear zone of β-type haemolysis); ++, moderate (medium 2-3 mm, clear zone of β-type haemolysis); +, weak reaction (clear but small zone 1 mm of β-type haemolysis); and - negative (no haemolysis or non-β-type haemolysis) (Wong et al., 1992).

4.2.2 Phospholipase assay

The phospholipase activity of V. parahaemolyticus was measured by inoculating isolates onto a phospholipase assay medium containing peptone, NaCl, agar and egg yolk emulsion (Appendix I). A clear lytic halo around the colonies indicated phospholipase activity. This method was a modification of Sudheesh and Xu, (2001) who used 25 µl of ECP and placed it in wells cut in agarose that contained 2.5% egg yolk.

4.2.3 Urease assay

Urease activity was observed on urea agar base plates containing peptone, NaCl, Na₂HPO₄, glucose, KH₂PO₄, phenol red, agar and urea solution (Appendix I). Production of urease was determined by a pink colour (alkaline) to the medium. Release of ammonia due to urease activity was detected by a change of pH indicator colour from yellow to pink (Atlas, 1997).
4.2.4 Protease assay

Protease activity was measured on a skim milk agar medium containing skim milk, NaCl and agar (Appendix I) (Wong et al., 1992). The positive results were recorded when a clear halo was detected around the growth.

4.2.5 Lipase assay

The lipase assay medium containing peptone, NaCl, CaCl$_2$, H$_2$O, agar and tween 80 (Appendix I) was used to determine the lipase activity by incubating at 37°C for 24-48 h (Wong et al., 1992). A positive result was indicated by a white precipitation around colonies.

4.2.6 *V. parahaemolyticus* putative virulence genes *tdh* and *trh*

Data of PCR targeting *tdh* and *trh* genes were provided by A. Miah, PhD thesis, University of Plymouth, and C. Baker-Austin, CEFAS Laboratories, Weymouth, Dorset, UK.

**Table 4.1 Sequences of previously described PCR primers for putative *V. parahaemolyticus* virulence gene *tdh* and *trh.*

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence gene (5’ to 3’)</th>
<th>Reference</th>
<th>Target genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tdh-D3</em></td>
<td>F: CCACTACCACCTCTCATATGC</td>
<td>Tada et al., (1992)</td>
<td><em>tdh</em></td>
</tr>
<tr>
<td><em>Tdh-D5</em></td>
<td>R: GGTACTAAATGGCTGACATC</td>
<td>Tada et al., (1992)</td>
<td><em>tdh</em></td>
</tr>
<tr>
<td><em>Trh-R2</em></td>
<td>F: GGCTCAAATGGTTAAGCG</td>
<td>Tada et al., (1992)</td>
<td><em>trh</em></td>
</tr>
<tr>
<td><em>Trh-R6</em></td>
<td>R: CATTTCCGCTCTCATATGC</td>
<td>Tada et al., (1992)</td>
<td><em>trh</em></td>
</tr>
</tbody>
</table>
4.2.7 Screening for plasmids

All isolates of *V. parahaemolyticus* were screened for plasmid DNA according to two procedures:

1- Bimboim and Doly, (1979); a single colony from overnight bacterial plate culture was grown in 5 ml TSB with shaking overnight at 37 °C. The organisms in 1.5 ml of the culture were harvested by centrifugation at 2,400 x *g* for 2 min and the cell pellet resuspended in 100 µl of B&D solution I (Appendix I). Next, 200 µl of B&D solution II (Appendix1) was added, mixed by gentle inversion and the tube left at room temperature for 5 min. Then 150 µl of ice-cold B&D solution III (Appendix I) was added and the tubes shaken gently and left on ice for 5 min. To yield a fresh supernatant, the tubes were then centrifuged at 9,500 x *g* for 3 min. Next, 400 µl of the supernatant was transferred to a clean 1.5 ml Eppendorff tube. Plasmids were precipitated by the addition of 1 ml of ice-cold absolute ethanol and placed on ice for at least 5 min. Following centrifugation for 3 min at 9,500 x *g* to collect precipitated nucleic acids, the supernatant was discarded and the tube placed upside-down on a piece of tissue for a few minutes to drain the pellet of ethanol. The dried pellet was resuspended in 10 µl TE buffer (Appendix I). Extracted plasmid DNA was electrophoresed at 90 V in 0.7 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). A 1 kilobase-ladder nucleic acid molecular weight standard (Invitrogen, UK) was included on each gel to confirm successful running of the gel. The plasmids were visualised using a UV transilluminator (Photodyne), and were photographed by UV light (309 nm) with a Gel Documentation system (Uvi-Tech, UK). This method was particularly suitable for screening for smaller sized plasmids. The approximate plasmid sizes were estimated by comparing with
supercoiled DNA ladder. Plasmids (pUC18, pBR322 and pBR328) with known molecular sizes (2.7 kb, 4.2 kb and 4.8 kb) respectively were extracted and used as control positive for the plasmid extraction procedure.

2- Pedraza and Díaz Ricci, (2002), the cell pellets were harvested in the same way as above and washed twice by gently suspending to safe avoid a premature cell destruction in cold 0.3 % Sodium Lauroyl Sarcosinate (sarkosyl) in TBE buffer and then centrifuged 2,400 x g for 7 min. A 20 µl of lysis solution (Appendix I) was used to suspend the pellet, and mixed with 30 µl of loading buffer and the agarose gel (0.7%) was prepared with 1 % SDS and run as above but at 60 V. This method was used to search for larger sized plasmids.

4.2.8 Preparation of extracellular products (ECPs)

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 24 - 48 h with shaking. The cultures were centrifuged at 2,800 x g for 15 min, and the supernatants were transferred to a fresh tube and re-centrifuged as before. Minicon B15 Clinical Sample Concentrators (Millipore, USA) were used to concentrate the supernatants according to the manufacturer’s instructions. All stages were performed at 4°C to reduce sample degradation.

4.2.9 Examination for the presence of bacteriophages

A 400 µl aliquot of an overnight broth culture of each the appropriate V. parahaemolyticus isolates was added separately to 5 ml molten soft Tryptone Soy agar, and used to overlay TSA plates. The concentrated supernatant from all isolates were tested for the presence of bacteriophage by spotting 2-4 µl on to
surface of an agar plate overlaid separately with each of the *V. parahaemolyticus* isolates, and plates were incubated overnight at 37 °C.

### 4.2.10 Preparation of whole cell proteins

Each bacterial isolate was grown in TSB (10 ml) at 37°C for 24 - 48 h, and centrifuged at 2,800 x g for 10 min at 4 °C. Pellets were washed once in PBS and resuspended in 500 µl of SDS-PAGE sample loading buffer (Appendix I), boiled for 5 min (to denature and solubilise the proteins) and centrifuged at 2,400 x g to remove insoluble cell debris. Approximately 16 µl of the sample was taken and subjected to SDS-polycrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

### 4.2.11 SDS-PAGE

The cell pellets and ECPs were analysed by SDS-PAGE using the discontinuous buffer system of Laemmli, (1970). In this project, the mini-protein II system (Bio-Rad, Herts, UK) was used as electrophoresis apparatus.

#### 4.2.11.1 Gel preparation

Spacers were placed on to one glass plate and the rubber gasket was fixed. The second glass plate was placed on top and all was clipped together. All solutions and composition of the gel used here are given in Appendix I. The bis-acrylamide (12.5%) was used as a uniform separating gel. All the components of the gel were mixed together except \( N,N,N',N' \)-tetramethylethylenediamine (TEMED) and 10% ammonium peroxodisulfate (APS) (must be fresh) which were added at the end. The gel mix was poured into the gel former and water saturated isobutanol was added gently on the top of the separating gel to exclude air and promote gel polymerisation and to give a very sharp gel-liquid interface. Polymerisation of the separating gel took about 45 min. To remove traces of isobutanol, the surface of
the gel was rinsed with distilled water before pouring the stacking gel (5%). A plastic comb was inserted into the gel prior to polymerisation to form sample wells.

4.2.11.2 Electrophoresis conditions

The comb and elastic gasket spacer were removed and the wells were rinsed with distilled water. The gel sandwich was placed into the electrophoresis apparatus and the tank filled with electrode buffer taking care to eliminate bubbles from the bottom of the gel. Samples were loaded into the wells and electrophoresis was performed at room temperature using constant voltage 125 V. Electrophoresis was stopped when the tracking dye was about one cm from the bottom of the gel. A ColorBurst™ Electrophoresis Marker (M.W. 8,000-220,000) (Sigma, UK) was used to estimate the molecular weight of protein bands. The gel apparatus and the glass sandwich were disassembled. The glass plates were gently prized apart by using the spacer as a lever. The gel was transferred on one plate to a container filled with staining solution.

4.2.11.3 Staining of SDS-PAGE gels and destaining

The gels were stained with Coomassie brilliant blue R-250 (Fisher Scientific Ltd., UK) (Appendix I). Gels were stained overnight at ambient temperature with gentle agitation. Gels were destained using destaining solution (Appendix I) with gentle agitation being exchanged every half an hour until protein bands were resolved.

4.2.12 Assessment of cell viability of *V. parahaemolyticus*

This a quick test that was carried out to assess if ECPs caused the cell damage or if apparent ECPs were as a result of autolysis. To assess *V. parahaemolyticus* cell viability, the cultures were grown on Tryptone soya broth (TSB; Oxoid, Basingstoke, UK) supplemented with 3% (w/v) sodium chloride at 37 °C for 24 h
and 48 h. An equal volume (200 µl) of 0.4% trypan blue was added to 200 µl of cell suspension (24 h and 48 h), the mixture were incubated at room temperature for 3 min and loaded into a haemocytometer. The non-viable (deep blue cells) and viable (clear cells) were counted. Viability of cells was calculated as follow: 

\[ \% \text{ viable} = \frac{\text{number viable cells}}{\text{number total cells}} \times 100 \]

### 4.2.13 Statistical analysis

Data were compared statistically using the Chi-Square (\( \chi^2 \)) test. The relationships between Kanagawa phenomenon and \( tdh \) gene; urease and \( trh \) gene were assessed by correlation; the correlation coefficient “r” is a qualitative measure of association and relationship between variables. The relationship between the plasmid and putative virulence factors was also investigated. Statistical analyses were performed using Minitab 16.0 software (Plymouth University, UK). (Appendix II).
4.3 Results

4.3.1 Assay of Kanagawa haemolysis activity

Kanagawa haemolysis activity on Wagatsuma agar was assessed in all 55 isolates of *V. parahaemolyticus*. KP\(^+\) tests were obtained in 82.60% and 15.62% which were from clinical and environment *V. parahaemolyticus* isolates, respectively. KP\(^+\) rates were significantly different in isolates from these two sources (*p* < 0.001) (Table 4.3).

However, *V. parahaemolyticus* showed varying degrees of haemolysis as indicated in Table 4.2. Of all isolates tested, only 5 isolates of clinical *V. parahaemolyticus*, showed strong haemolysis (indicated +++). Moderate (++) and weak (+) haemolysis were exhibited in 10 isolates (7 clinical, 3 environmental) and 9 isolates (7 clinical, 2 environmental) respectively. However, there was no haemolytic activity in 31 isolates, of which 4 were clinical, and 27 environmental.

A selection of *Vibrio* species isolates were tested for Kanagawa haemolysis, the results showed that *V. alginolyticus* (VA3) exhibited moderate haemolysis, *V. vulnificus* (VV2) exhibited weak haemolysis, while there was no haemolysis in *V. cholerae* (VC), *V. mimicus* (VM), *V. alginolyticus* (VA6), *V. harveyi* (VH4) and *V. anguillarum* (VN1) (Table 4.4).

4.3.2 Assay of enzymatic activities

All the clinical *V. parahaemolyticus* isolates and 15 environmental isolates were able to hydrolyse urea. Some isolates exhibited positive results after 24 h. While other isolates showed urease positive after two days (Table 4.2).

However, *V. parahaemolyticus* isolates that hydrolyse urea rates in clinical and environment *V. parahaemolyticus* isolates were 100 % and 46.87%, respectively.
Urease positive rate in clinical isolates was significantly different from environmental isolates ($p < 0.001$) (Table 4.3).

Four *Vibrio* species isolates tested including *V. cholerae* (VC), *V. mimicus* (VM), *V. alginolyticus* (VA3) and *V. vulnificus* (VV2) exhibited positive urease after 48 h, while *V. alginolyticus* (VA6), *V. harveyi* (VH4) and *V. anguillarum* (VN1) did not show any urease activity (Table 4.4).

All isolates were also tested for phospholipase activity, it has been found that the phospholipase positive in clinical and environmental isolates were 82.60% and 15.62%, respectively. Phospholipase positive rate in clinical isolates was significantly different from environmental isolates ($p < 0.001$). *V. cholerae* (VC) and *V. vulnificus* (VV2) exhibited phospholipase activity, while other *Vibrio* species tested did not.

Protease activity was tested for all *V. parahaemolyticus* isolates, the results showed that the protease positive in clinical and environmental isolates were 82.60% and 43.75%, respectively. Protease positive rates in isolates from these two sources were significantly different from each other ($p < 0.01$). Amongst of a selection of *Vibrio* species, only *V. vulnificus* (VV2) and *V. alginolyticus* (VA3) showed protease activity.

Lipase activities were obtained in 26 % and 15.62% which were from clinical and environment *V. parahaemolyticus* isolates, respectively. Lipase positive rates were not significantly different in isolates from these two sources ($p$-value = 0.339). All selection of *Vibrio* species isolates tested did not show lipase activity.
Correlation of specific phenotypes and genotypes

a) Correlation of KP test and \textit{tdh} gene:

The Pearson correlation-coefficient was high (\(r = 0.741, p = 0.0001\)).

b) Correlation of urease test and \textit{trh} gene:

The Pearson correlation-coefficient was low (\(r = 0.280, p = 0.03\)).

4.3.3 Investigation into the presence of bacteriophages in \textit{V. parahaemolyticus}

All isolates of \textit{V. parahaemolyticus} were tested for bacteriophages. No bacteriophages were found in any of the isolates, using the simple method employed.

4.3.4 Investigation into the presence of plasmids in \textit{V. parahaemolyticus}

Among the clinical isolates, 8 out of 23 (C17, C19, C16, C21, R1, C1, C7 and C14) (34.78\%) had plasmids. Likewise, 10 out of 32 environmental isolates (E1, E30, E31, E7, E10, E2, E26, E9, E5, and E19) (31.25\%) were found to have plasmids. Some possessed multiple plasmid bands (Figure 4.1). The approximate sizes and number of plasmid were indicated in Table 4.5.

Based on the geographical origin, two out of ten (20\%) isolates from Norway had different size of plasmids, one out of four (25\%) isolates from Japan had two plasmids, seven out of 25 (28\%) isolates from UK had different size of plasmids, except two environmental isolates (E9 and E19) had similar size at approximately 16 kb. Amongst Spanish isolates, 6 (3 clinical and 3 environmental) out of 11 (54.54\%) isolates had different size of plasmids except two clinical isolates (C17 and C19) had similar size at approximately 2.6 kb, however, clinical isolate C16
had multiple plasmids at approximately 3.6; 6.6; 26.5; 70 kb, and E2 had two plasmids at approximately 14 and 75 kb.

**Correlations of plasmid and putative virulence factors**

The relationship between the plasmid and putative virulence factors such as KP, urease, *tdh* and *trh* genes was investigated.

a) Correlation of plasmid and KP test:

There was no correlation ($r = 0.011$, $p = 0.934$).

b) Correlation of plasmid and *tdh* gene:

There was no correlation ($r = -0.120$, $p = 0.383$).

c) Correlation of plasmid and urease activity:

There was no correlation ($r = -0.120$, $p = 0.381$).

d) Correlations of plasmid and *trh* gene:

There was no correlation ($r = -0.206$, $p = 0.132$).

**4.3.5 Whole cell protein profiles**

Initially, whole cell protein samples of two isolates of *V. parahaemolyticus* reference strain (R1) and clinical isolate (C5) were applied into SDS-PAGE with different amounts (2µl, 4µl, 8µl, 10µl, 12µl, 16µl, 18µl and 20µl) in order to determine how much was needed to use to get comparable intensity of bands (Figure 4.2). Whole cell protein samples of additional four isolates (C1, C7, E16 and C4) were also applied into SDS-PAGE with different amounts (Appendix II). The results showed that 16 µl was suitable amount to show clearly all the bands.

Then, 16 µl of whole cell protein samples of all isolates (23 clinical and 32 environmental) were applied into SDS-PAGE to reveal protein profiles of isolates tested. The results of SDS-PAGE of whole cell proteins showed many discrete
bands ranging in molecular weight of 8 kDa to 220 kDa. All isolates had many common bands (Figure 4.3). Moreover, whole cell protein patterns of clinical *V. parahaemolyticus* isolates were generally similar to environmental *V. parahaemolyticus*. There were no obvious differences between clinical and environmental isolates; but there were minor differences in the number of bands produced in some isolates, for example there was extra band at 90 kDa found in only four environmental isolates (E12, E13, E14 and E15). A band at 60 kDa was detected in only one clinical (C1) and one environmental (E16) isolates.

### 4.3.6 ECPs profile

The ECP profile was also examined by SDS-PAGE. The ECPs of reference strain of *V. parahaemolyticus* (R1) was harvested at different times (2h, 4h, 8h, 16h, 24h, 32, 40h and 48h). As shown in Figure 4.4, there was a very faint band detected at 41 kDa after 16h, while this band was clearly detected at 24 h. Another band at 40 kDa was detected in 32 h. However, about 7-11 bands were found in ECPs harvested from 40 and 48 h cultures, however, the bands that shown in 48 h were clearer than in 40 h.

#### 4.3.6.1 ECPs harvested from 24 hours cultures

Comparative protein bands of the ECPs of 23 clinical and 32 environmental isolates of *V. parahaemolyticus* that were harvested in 24 hours incubation of the culture at 37°C were observed by SDS-PAGE. The ECPs exhibited diversity among the isolates; however, most shared a band at 41 kDa that was produced by 11 clinical (C7, C5, C14, C12, R1, C2, C8, C9, C11, C17 and C18) isolates and one environmental isolate (E3). Two clinical isolates (C3 and C6) produced this band but it was very faint. However, a few strains did not produce any band (Figure 4.5).
4.3.6.2 ECPs harvested from two day cultures

The ECPs of 23 clinical and 32 environmental isolates of *V. parahaemolyticus* exhibited diversity between the isolates. The ECPs of most isolates generated less than 12 bands, ranging from 10-190 kDa. The ECP of five clinical isolates (C8, C1, C13, C5, and C7) and two environmental isolates (E4 and E15) generated a prominent band at 60 kDa.

A protein band at 41 kDa was found in 14 clinical isolates (C2, C8, C9, C11, R1, C1, C12, C13, C14, C15, C3, C4, C5, C7), but not in all clinical isolates; and only in two environmental isolates (E3 and R2). Different protein profiles were observed among the remainder of clinical and environmental isolates of *V. parahaemolyticus* tested Figure 4.6.

4.3.7 The viability of bacterial cells

The results showed that the percentage of viability of *V. parahaemolyticus* cell in triplicate was 77% and 66.5% in 24 h and 48 h consequently.
Table 4.2 Kanagawa haemolysis of *V. parahaemolyticus* on Wagatsuma agar and urease activity of *V. parahaemolyticus* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type and source (where known)</th>
<th>Kanagawa haemolysis</th>
<th>Urease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt; 3 mm</td>
<td>2-3 mm</td>
</tr>
<tr>
<td>R1</td>
<td>Clinical, NCIMB Reference Strain, UK</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>Clinical, Norway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>Clinical, Norway</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>Clinical, Norway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>Clinical, Norway</td>
<td>+++</td>
<td></td>
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<tr>
<td>C5</td>
<td>Clinical, Norway</td>
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<tr>
<td>C6</td>
<td>Clinical, Norway</td>
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<td>+</td>
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<td>C8</td>
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<td>C9</td>
<td>Clinical, Norway</td>
<td>++</td>
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<td>C10</td>
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<td>C11</td>
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<td>+</td>
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<td>C12</td>
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<td>++</td>
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<tr>
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<td>C16</td>
<td>Clinical, Spain</td>
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</tr>
<tr>
<td>C17</td>
<td>Clinical, Spain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18</td>
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<td>++</td>
<td></td>
</tr>
<tr>
<td>C19</td>
<td>Clinical, Spain</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>C20</td>
<td>Clinical, Spain</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>C21</td>
<td>Clinical, Italy</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C22</td>
<td>Clinical, UK</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>Environmental, NCTC Reference Strain, UK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>Environmental, Spain</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>Environmental, Spain</td>
<td>-</td>
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</tr>
<tr>
<td>E4</td>
<td>Environmental, Spain</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E5</td>
<td>Environmental, Spain</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>Environmental, Spain</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E7</td>
<td>Environmental, UK</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>E8</td>
<td>Environmental, UK</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E9</td>
<td>Environmental, UK</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>E10</td>
<td>Environmental, UK</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E11</td>
<td>Environmental, UK</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>E12</td>
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<td>-</td>
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</tr>
<tr>
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<tr>
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<td>Environmental, UK</td>
<td>-</td>
<td></td>
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<td>Environmental, UK</td>
<td>-</td>
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<td>E17</td>
<td>Environmental, UK</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E18</td>
<td>Environmental, UK</td>
<td>+</td>
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</tr>
<tr>
<td>E19</td>
<td>Environmental, UK</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E20</td>
<td>Environmental, UK</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>E21</td>
<td>Environmental, UK</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E22</td>
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</tr>
<tr>
<td>E23</td>
<td>Environmental, UK</td>
<td>-</td>
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<td>E24</td>
<td>Environmental, UK</td>
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<tr>
<td>E25</td>
<td>Environmental, UK</td>
<td>-</td>
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</tr>
<tr>
<td>E26</td>
<td>Environmental, UK</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E27</td>
<td>Environmental, Portugal</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E28</td>
<td>Environmental, Portugal</td>
<td>-</td>
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</tr>
<tr>
<td>E29</td>
<td>Environmental, UK</td>
<td>-</td>
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</tr>
<tr>
<td>E30</td>
<td>Environmental, UK</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E31</td>
<td>Environmental, UK</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

(9.09%) (18.18%) (16.36%) (56.36%) 29.09% 40% 30.90%

+++ Strong; ++, moderate; +, weak; -, negative.
Table 4.3 Kanagawa Phenomenon and enzyme activities of *V. parahaemolyticus*

<table>
<thead>
<tr>
<th>Isolated source</th>
<th>Total</th>
<th>KP+ percentage</th>
<th>Ure+ percentage</th>
<th>Lip+ percentage</th>
<th>Pro+ percentage</th>
<th>PPL+ percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KP</td>
<td>Ure</td>
<td>Lip</td>
<td>Pro</td>
<td>PPL</td>
</tr>
<tr>
<td>Clinical</td>
<td>23</td>
<td>19 (82.60)</td>
<td>100</td>
<td>26</td>
<td>4 (82.60)</td>
<td>4 (82.60)</td>
</tr>
<tr>
<td>Environmental</td>
<td>32</td>
<td>5 (15.62)</td>
<td>46.87</td>
<td>27</td>
<td>18 (43.75)</td>
<td>27 (15.62)</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>24 (43.64)</td>
<td>38 (17.59)</td>
<td>17 (31.82)</td>
<td>22 (40.36)</td>
<td>22 (40.36)</td>
</tr>
</tbody>
</table>

For Kanagawa Phenomenon tests, $\chi^2 = 24.412, p < 0.001$; for Urease tests, $\chi^2 = 17.685, p < 0.001$; for Lipase tests, $\chi^2 = 0.915, p > 0.01$; for protease tests, $\chi^2 = 8.419, p < 0.01$; for phospholipase tests, $\chi^2 = 24.412, p < 0.001$.
Table 4.4 Kanagawa Phenomenon and enzyme activities of a selection of *Vibrio* species isolates

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Bacterial Strain</th>
<th>Virulence Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KP (β-haemolysis)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;3mm</td>
</tr>
<tr>
<td>VC</td>
<td><em>V. cholerae</em></td>
<td>-</td>
</tr>
<tr>
<td>VM</td>
<td><em>V. mimicus</em></td>
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</tr>
<tr>
<td>VV2</td>
<td><em>V. vulnificus</em></td>
<td>+</td>
</tr>
<tr>
<td>VA3</td>
<td><em>V. alginolyticus</em></td>
<td>++</td>
</tr>
<tr>
<td>VA6</td>
<td><em>V. alginolyticus</em></td>
<td>-</td>
</tr>
<tr>
<td>VH4</td>
<td><em>V. harveyi</em></td>
<td>-</td>
</tr>
<tr>
<td>VN1</td>
<td><em>V. anguillarum</em></td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.5 Putative virulence factors and genes, and plasmid possession of *Vibrio parahaemolyticus* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type and source</th>
<th>Virulence Characteristics and virulence genes</th>
<th>Plasmid DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(where known)</td>
<td>KP (β-haemolysis) Ure Lip Pro PPL tdh trh</td>
<td>Number of bands Approximate size of plasmid (kb)</td>
</tr>
<tr>
<td>R1</td>
<td>Clinical, NCIMB</td>
<td>+ - - + - - + (1)</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Reference Strain, UK</td>
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<td></td>
</tr>
<tr>
<td>C1</td>
<td>Clinical, Norway</td>
<td>+ + - + + -</td>
<td>+ (1)</td>
</tr>
<tr>
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</tr>
<tr>
<td>C6</td>
<td>Clinical, Norway</td>
<td>+ + - + + -</td>
<td>+ (2)</td>
</tr>
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</tr>
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<td>C8</td>
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<td>+ (2)</td>
</tr>
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<tr>
<td>C16</td>
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<td>+ + - + + -</td>
<td>+ (4)</td>
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<tr>
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<td>- - - + + -</td>
<td>+ (1)</td>
</tr>
<tr>
<td>C18</td>
<td>Clinical, Spain</td>
<td>+ + - + + -</td>
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</tr>
<tr>
<td>C19</td>
<td>Clinical, Spain</td>
<td>+ + - + + -</td>
<td>+ (1)</td>
</tr>
<tr>
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<td>+ + - + + -</td>
<td></td>
</tr>
<tr>
<td>C21</td>
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<tr>
<td>C22</td>
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</tr>
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<td>+ (1)</td>
</tr>
<tr>
<td>E2</td>
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<td>+ (2)</td>
</tr>
<tr>
<td>E3</td>
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<tr>
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<td>E5</td>
<td>Environmental, Spain</td>
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<td>E6</td>
<td>Environmental, Spain</td>
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<td></td>
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<tr>
<td>E7</td>
<td>Environmental, UK</td>
<td>+ - - + + -</td>
<td>+ (1)</td>
</tr>
<tr>
<td>E8</td>
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<td>+ (1)</td>
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<td>+ (3)</td>
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<tr>
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<tr>
<td>E12</td>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
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<td>E16</td>
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<tr>
<td>E18</td>
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<td>+ (1)</td>
</tr>
<tr>
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<tr>
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<tr>
<td>E23</td>
<td>Environmental, UK</td>
<td>+ - - - - -</td>
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<td>Environmental, UK</td>
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<td>+ (2)</td>
</tr>
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<td>E28</td>
<td>Environmental, Portugal</td>
<td>- - - - - -</td>
<td></td>
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<tr>
<td>E29</td>
<td>Environmental, UK</td>
<td>+ - - - - -</td>
<td></td>
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<tr>
<td>E30</td>
<td>Environmental, UK</td>
<td>+ - - - - -</td>
<td>+ (4)</td>
</tr>
<tr>
<td>E31</td>
<td>Environmental, UK</td>
<td>+ - - - - -</td>
<td>+ (1)</td>
</tr>
</tbody>
</table>

(1) Virulence characteristics, KP: Kanagawa phenomenon, Ure: urease, Lip: lipase, Pro: protease and PPL: Phospholipase activities and data of PCR virulence genes (*tdh* and *trh*) provided by A. Miah (Plymouth University) and C. Baker-Austin, CEFAS Laboratories, Weymouth, Dorset, UK.
Figure 4.1 Agarose gel electrophoresis of plasmid from *V. parahaemolyticus*. Lanes correspond to the following M$, \text{1Kb linear DNA ladder; M}^2$ and M$, \text{supercoiled DNA ladders}; 1, E21; 2, E30; 3, R2; 4, C12; 5, C17; 6, E31, 7, C19; 8, C22; 9, C16; 10, C18; 11, C21; 12, E29; 13, E28; 14, E3; 15, C11; 16, C6; 17, C8; 18, C5; 19, E27; 20, E25; 21, E4; 22, E12; 23, C14; 24, C15; 25, E16; 26, E9; 27, E19; 28, E20; 29, E5; 30, E1; 31, E15; 32, E13; 33, E22; 34, E17; 35, E10; 36, E2; 37, E23; 38, E24; 39, E26; 40, R1; 41, C1; 42, C7; 43, C9; 44, C13; 45, E7; 46, E14; 47, C20; 48, C2; 49, C3; 50, C4; 51, C10; 52, E6; 53, E8; 54, E18; 55, E11, 56, control negative; lanes 57-60, control positive as follow, 57, pUC18; 58-59, pBR322; 60, pBR328.
Figure 4.2 SDS-PAGE of soluble whole cell proteins from clinical *V. parahaemolyticus* isolates with different concentrations.

M, protein marker (M.W. = 8 kDa-220 kDa).

a, Clinical reference strain (R1)

b, Clinical isolate (C5)
Figure 4.3 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, E11; 4, E4; 5, E5; 6, E2; 7, E1; 8, R2; 9, C3; 10, C4; 11, C5; 12, C7; 13, E8; 14, E22; 15, E19; 16, E13; 17, E14; 18, E3; 19, E6; 20, E7; 21, E12; 22, E15; 23, E16; 24, E25; 25, C15; 26, E24; 27, E26; 28, R1; 29, C1; 30, C6; 31, E17; 32, E21; 33, E18; 34, E23; 35, E20; 36, C9; 37, E9; 38, E10; 39, C10; 40, C11; 41, C12; 42, C13; 43, C14; 44, C16; 45, C17; 46, C18; 47, C19; 48, C20; 49, C21; 50, C22; 51, E27; 52, E28; 53, E29; 54, E31; 55, E30.
Figure 4.4 SDS-PAGE of ECPs of reference strain of *V. parahaemolyticus* (R1) in different times. M, protein marker (M.W. = 8 kDa to 220 kDa).
Figure 4.5 SDS-PAGE of ECPs from *V. parahaemolyticus* isolates (one day).

**M**, protein marker (**M.W. =**8 kDa-220 kDa), 1, C7; 2, C5; 3, E3; 4, E4; 5, E5; 6, E2; 7, R2; 8, C3; 9, C4; 10, E10; 11, E11; 12, E8; 13, C14; 14, C12; 15, E9; 16, E6; 17, E7; 18, E12; 19, E15; 20, E16; 21, C21; 22, E30; 23, E29; 24, E27; 25, E28; 26, E31; 27, C2; 28, C1; 29, C6; 30, C13; 31, E21; 32, E18; 33, E23; 34, E20; 35, R1; 36, C8; 37, C9; 38, C10; 39, C11; 40, E14; 41, E17; 42, E13, 43, C15; 44, E24; 45, E26; 46, E25; 47, E1; 48, E22; 49, E19; 50, C16; 51, C17; 52, C18; 53, C19; 54, C20; 55, C22.
Figure 4.6 SDS-PAGE of ECPs from *V. parahaemolyticus* isolates (two days).

**M**, protein marker (M.W. = 8 kDa-220 kDa), 1:E11, 2:E10, 3:E3, 4:E4, 5:E5, 6:E2, 7:E1; 8, R2; 9, C3; 10, C4; 11, E13; 12, E14; 13, E8; 14, E12; 15, E19; 16, C5; 17, C7; 18, E9; 19, E6; 20,E7; 21, E22; 22, E15; 23, E16; 24, E25; 25, C15; 26, E24; 27, E26; 28, R1; 29, C1; 30, C6; 31, C12; 32, C13; 33, C14; 34, E23; 35, E20; 36, C2; 37, C8; 38, C9; 39, C10; 40, C11; 41, E17; 42; E21, 43, E18; 44, C21; 45, E30; 46, E29; 47, E27; 48, E28; 49, E31; 50, C16; 51, C17; 52, C18; 53, C19; 54, C20; 55, C22.
4.4 Discussion:

4.4.1 Assay of Kanagawa haemolysis activity

The percentages of strains of *V. parahaemolyticus* with haemolytic activity on Wagatsuma agar were negative 56.36%, weak 16.36%, moderate 18.18% and strong 9.09%. The results of this study can be compared with Wong et al., (1992) who studied haemolytic activity of *V. parahaemolyticus* isolated from seafood samples, and they found that the percentages of haemolytic activity were negative 34.5%, weak 53.4%, moderate 3.9%-4.9% and strong 3.4%.

In the present study, Kanagawa haemolysis activity was detected in 82.6% clinical isolates and 15.6% environmental isolates. The result for clinical isolates is supported by Hongping et al., (2011) who have isolated *V. parahaemolyticus* from clinical cases, fresh water and seafood, and detected KP as a positive result in 88.66% of clinical isolates, but also in 76.67% of isolates from fresh water and 46.67% isolates from seafood in China. Furthermore, Nishibuchi and Kaper (1995) have reported 90% of clinical cases exhibited β-haemolysis on Wagatsuma agar, and found about ≥2% of environmental isolates exhibited KP+. Kelly and Stroh (1989) have found that about 1% of environmental *V. parahaemolyticus* strains are KP+. The KP has not always been associated with the production of TDH (Nishibuchi and Kaper, 1985). Nowadays, the KP reaction is usually used as a marker for detecting pathogenic *V. parahaemolyticus* (Honda and Iida, 1993, Izutsu et al., 2008). However, Hondo et al., (1987) have recorded that KP− strains may also cause gastroenteritis.

In the current study, although KP was associated with *tdh* gene, one clinical (C17) and two environmental (E14 and E21) that bore the *tdh* gene were found to be negative for KP. Suthienkul et al., (1995) who also demonstrated that some clinical
*V. parahaemolyticus* isolates having *tdh* rarely exhibit haemolytic reaction on Wagatsuma agar even if those strains have the *tdh* gene, probably because the *tdh* gene is not expressed.

Conversely, three clinical (R1, C14 and C21) and one environmental (E11) 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 (Nishibuchi et al., 1985, Hoashi et al., 1990). Therefore, other factors may contribute to haemolytic activity (Izutsu et al., 2008). Another possibility is that the haemolysis might be caused by phospholipase activity as demonstrated by other bacterial species (Kreger et al., 1987).

Furthermore, Chun et al., (1975), Honda et al., (1980) and Nishibuchi et al., (1985) have reported that there are many limitations with KP test, including difficulty of interpretation in some isolates, NaCl concentration, erythrocyte type used in Wagatsuma agar and pH variability.

Recently, Rojas et al., (2011) have recorded that an environmental isolate which does not carry the *tdh* gene still produced haemolytic activity on Wagatsuma agar (KP⁺). The contribution of TDH to pathogenicity of *V. parahaemolyticus* remains unclear (Hiyoshi et al., 2010). Moreover, Hoashi et al., (1990) and Honda et al., (1983) have suggested that there are other virulence factors associated with pathogenicity of this bacterium. Canizalez-Roman et al., (2011) have demonstrated that the best incubation time to interpret the results of KP was at 48 h; and furthermore, noted that KP is inaccurate and not the best tool to detect all virulent forms of *V. parahaemolyticus*. 
4.4.2 Phospholipase activity

Phospholipases can be virulence factors for many bacterial species that cause infection such as *Pseudomonas aeruginosa, Listeria monocytogenes, Clostridium perfringens* and *Corynebacterium pseudotuberculosis* (Songer, 1997). Furthermore, this enzyme has been considered as a virulence factor in many *Vibrio* species such as *V. cholerae* (Fiore et al., 1997). Therefore, in current work, all isolates were tested on egg yolk plates to assay phospholipase activity; 82.60% of clinical isolates of *V. parahaemolyticus* and 15.62% environmental isolates showed phospholipase activity. These results suggest that *V. parahaemolyticus* produces a phospholipase that might be involved in pathogenesis, and are supported by the finding of Guhathakurta et al., (1988) who demonstrated that phospholipase plays a role as a virulence factor in pathogenicity of *V. parahaemolyticus*.

Other bacterial studies reported that phospholipase is an important virulence factor associated with pathogenicity, for example, *Legionella* species produces phospholipase A that contributes to severe pneumonia by destroying alveolar surfactant phospholipid in the lung (Flieger et al., 2000a, Flieger et al., 2000b). Amongst human pathogenic *Vibrio* species, phospholipase A plays a role in the pathogenesis of *V. mimicus* infection (Lee et al., 2002b), and *V. vulnificus* infection (Koo et al., 2007).

In the current study, of the 24 phospholipase positive isolates, 20 were also KP+ isolates, suggesting that there is a relationship between these two activities. Phospholipase acts as a haemolysin in members of *Vibrionaceae* that produce and secreted this enzyme (Taniguchi et al., 1985, Thornton et al., 1988). A number of authors have reported some relationship between haemolytic activity and
phospholipase activity in some vibrios, for example, toxigenic *V. cholerae* O139 show phospholipase activity (Pal et al., 1997), furthermore, Shinoda et al., (1991) have reported that lecithin-dependent haemolysin of *V. parahaemolyticus* have phospholipase A₂/lysophospholipase. Zhong et al., (2006) have found that there is a strong relationship between haemolysin of *V. harveyi* that plays a role in pathogenicity in fish and phospholipase activity. Moreover, some *Vibrio* phospholipases such *V. vulnificus* and *V. mimicus* demonstrate haemolytic activity (Testa et al., 1984, Lee et al., 2002b). Sun et al., (2007) have demonstrated that a mutation at the active site of phospholipase of *V. harveyi* haemolysins was responsible for the loss of haemolytic and phospholipase activities and its virulence in fish, suggesting that phospholipase enzyme might be played an important role in haemolysis and pathogenicity. Furthermore, phospholipase associates with different pathogenic process and contributes to intestinal destruction (Gustafson and Tagesson, 1990, Titball, 1993). 

Further studies are needed to identify the role of *V. parahaemolyticus* phospholipase in infection and disease. Furthermore, investigations will be needed to determine if phospholipase alone can lyse erythrocytes.

### 4.4.3 Urease activity

Urease is defined as an enzyme from bacteria, fungi or plants that catalyses the hydrolysis of urea to carbon dioxide and ammonia (Olivera-Severo et al., 2006). Urease activity contributes to the inhibition of mucus biosynthesis. It causes intestinal mucus to disassemble at the mucosal surface facilitating bacterial colonisation and possibly the formation of ulcers (Sidebotham and Baron, 1990). Releasing a high concentration of ammonium ions, the urease enzyme may exert
toxic effects at intercellular junctions leading to alterations in permeability of intestine mucosa (Hazell and Lee, 1986).

The ability of bacteria to hydrolyse urea can be used to predict potential pathogenicity of these bacteria (Kelly and Stroh, 1989). Cai and Ni, (1996) have purified the enzyme from urease positive *V. parahaemolyticus* and tested it *in vivo*. The authors found that this enzyme caused intestinal fluid accumulation, suggesting that urease enzyme may play a role as a virulence factor and can be used as an indicator of pathogenicity. However, Kelly and Stroh (1989) have detected urease activity in clinical and environmental *V. parahaemolyticus* isolates that were negative for the KP reaction.

In the present study, urease activity was tested on urea agar base plates. All clinical isolates of *V. parahaemolyticus* and 46.87% of environmental isolates produced this enzyme and the correlation between urease enzyme and *trh* gene was low. Park, (2004d) has reported that the activity of urease-positive *V. parahaemolyticus* was not related to the carriage of haemolysin genes. Conversely, the clinical strains of *V. parahaemolyticus* that produced urease correlated with the presence the *trh* gene (Park et al., 2000). Moreover, in Korea, Kim and Kim, (2001) have reported that urease positive environmental *V. parahaemolyticus* strains carry *tdh* and *trh* genes, and suggested that urease is an important enzyme and correlated to pathogenicity of those strains. Recently, Sujeewa et al., (2009) tested 189 environmental *V. parahaemolyticus* isolates and found 41% exhibited urease positive. Furthermore, Honda, et al., (1992a) and Osawa, et al., (1996) also reported that no relationship exists with haemolysin genes and the ability of the isolates to hydrolyse urea, but it is possible to correlate with the *trh* gene. Moreover, not all urea positive isolates produce *tdh* and *trh*.
Of *Vibrio* species isolated from sea water, urease activity was found in 40% for *V. parahaemolyticus* and 40% for *V. cholerae* non-O1, 20% for *V. vulnificus* and 20% for *V. furnissii*, 5% for *V. alginolyticus*, 100% for *V. metshnikovii* and none for *V. fluvialis* (Baffone et al., 2001).

It is recommended in the future work to purify and characterise the urease enzyme from *V. parahaemolyticus* to identify its role in virulence and pathogenicity of *V. parahaemolyticus*.

### 4.4.4 Protease activity

Proteases are important enzymes which can be involved in pathogenicity and hydrolyse the peptide bonds in proteins to produce smaller molecules (Rao et al., 1998). Many *Vibrio* species produce protease enzymes that might contribute to the pathogenicity of those bacteria (Crowther et al., 1987, Chowdhury et al., 1990).

In the present study, protease activity was tested on skim milk agar medium. The results show that protease activity was detected in 82.60% of clinical and 40.62% of environmental isolates of *V. parahaemolyticus*. Park, (2004d) have found proteases in 43 out of 52 (82%) environmental strains, and suggested that *V. parahaemolyticus* isolated from environment had protease as a main pathogenic factor, but not haemolysin. Furthermore, Wong et al., (1992) have found 87.4% of environmental isolates were protease positive. Protease enzymes are considered virulence factors and contribute to the pathogenicity in some *Vibrio* species (Miyoshi and Shinoda, 2000). Ottaviani et al., (2005) have suggested that proteases were the main virulence factor in clinical *V. parahaemolyticus* isolates that did not carry *tdh* and *trh*. Proteins with proteolytic activity that are secreted and produced by pathogenic bacteria can have a role in tissue damage and pathogenesis. For example, the metalloprotease of *Helicobacter pylori* might
contribute to proteolysis of different host proteins \textit{in vivo} and thus is associated with pathogenesis to cause disease (Windle and Kelleher, 1997).

Protease activity has been tested in \textit{Vibrio} species isolated from seafood and aquacultured foods. Protease activity was found in 87.4\% for \textit{V. parahaemolyticus}, 78.2\% for \textit{V. alginolyticus}, 91.4\% for \textit{V. cholerae}, 90.9\% for \textit{V. fluvialis} I, 100\% for \textit{V. fluvialis} II, 93.3\% for \textit{V. mimicus}, 76.7 \% for \textit{Aeromonas hydrophila}, 69.2\% for \textit{A. caviae} and 79.2\% for \textit{A. sobria} (Wong et al., 1992).

Further investigation will be needed to determine if protease activity could contribute to pathogenicity by purifying this enzyme and testing \textit{in vivo} and \textit{in vitro}.

4.4.5 Lipase activity

Lipase is an enzyme produced and secreted by bacteria. It can catalyse both the synthesis and the hydrolysis of acylglycerols chain (Jaeger et al., 1999). Lipase activity was exhibited in six (26\%) clinical and five (15.62\%) environmental isolates of \textit{V. parahaemolyticus}. This enzyme is therefore not useful to differentiate the virulent and avirulent isolates of this bacterium.

However, lipases play a main role in pathogenesis of other bacteria such as \textit{A. hydrophila} and \textit{A. salmonicida} (Thornton et al., 1988). The lipase activity found in the present study did not correlate with haemolysis activity, as found by Zhong et al., (2006) in \textit{V. harveyi}. Ranjitha et al., (2009) have purified and characterised a lipase enzyme from \textit{V. fischeri} but generally, information about lipolytic enzymes produced by marine \textit{Vibrio} species is especially limited.

Studies about \textit{Vibrio} species isolated from sea water report that lipase activity was found in 100 \% for \textit{V. parahaemolyticus}, \textit{V. vulnificus}, \textit{V. alginolyticus}, \textit{V. fluvialis}, \textit{V. furnissii}, \textit{V. metshnikovii} and 80 \% for \textit{V. cholerae} non-O1 (Baffone et al.,
Furthermore, Wong et al., (1992) have tested lipase activity in *Vibrio* species isolated from seafood and aquacultured foods and lipase activity was found in 97.9% for *V. parahaemolyticus*, 98.1% for *V. alginolyticus*, 98.0% for *V. cholerae*, 95.7% for *V. fluvialis* I, 81.8% for *V. fluvialis* II, 97.7% for *V. mimicus*, 81.5% for *Aeromonas hydrophila*, 69.2% for *A. caviae* and 82.4% for *A. sobria*.

### 4.4.6 Plasmids and bacteriophages

Mobile genetic elements such as plasmids and bacteriophages can encode virulence factors and play a role in pathogenesis (Betley et al., 1986, Davis and Waldor, 2000). In the current study, *V. parahaemolyticus* isolates were screened by using agarose gel electrophoresis to check for the presence of plasmids which might carry genes for virulence factors. Various plasmids were found at similar frequency in clinical and environmental isolates, suggesting that there was no link between plasmid carriage and pathogenicity. However, regarding geographical regions; the incidence of plasmids was higher among isolates from Spain (54.54%) than the isolates from other countries such as Norway (20 %), UK (28%) and Japan (25%).

These results support those of Kelly and Stroh (1989) who found 15% and 13% of plasmid-bearing forms in clinical and environmental *V. parahaemolyticus* isolates, respectively, in the Pacific Northwest. Furthermore, Abbott et al., (1989) screened for plasmids in 16 *V. parahaemolyticus* isolates and about 30% were found to harbour such elements.

In Japan, the incidence of plasmids in *V. parahaemolyticus* has been studied by Arai, et al., (1983) who have found plasmids in 8 out of 20 clinical isolates, however, they did not find any plasmids in strains from foods. In Bangladesh no strains isolated from patients had plasmids; however, they found plasmids in two
out of eight environment isolates. Arai, et al., (1983) have suggested that fresh isolates may have more plasmids, and most of the plasmids in *V. parahaemolyticus* are unstable and segregate into small fragments in the course of stabilisation. Consequently, these plasmids may lose their biological markers through these changes.

Another study reported that there is no significant differences between plasmids isolated from clinical and environmental isolates of *V. parahaemolyticus*, thus the plasmid profiles method is not useful method for epidemiological studies (Kaufman et al., 2002). Depaola et al., (2003b) have used plasmids analysis to differentiate between virulent and avirulent *V. vulnificus* isolates; however, similar percentages have been found in clinical and environmental isolates of this organism.

In the current study, there was no correlation between carriage of plasmids and putative virulence factors. These plasmids appear not to be involved in virulence because some strains lacking plasmids were shown to be virulent, for example, 33.33% and 28.94% of KP+ and of urease positive *V. parahaemolyticus* had plasmids, respectively, suggesting that plasmids did not code for these factors, and that in the majority of the isolates virulence factors were not associated with the presence of plasmids. Guerry and Colwell (1977) reported plasmids detected in KP+ strains of *V. parahaemolyticus* could be involved in production of this haemolysin or other features that contributed with pathogenicity of these strains. Moreover, Kelly and Stroh (1989) found that KP+ *V. parahaemolyticus* did not carry any plasmids, and suggested that the presence of plasmids in this bacterium does not correlate with Kanagawa haemolysis.

In the present study, bacteriophages were not found in any culture supernatant of isolates, suggesting that bacteriophage if present may be integrated into the
bacterial genome. Phages have been found in \textit{V. parahaemolyticus} by other authors, for example, Iida et al., (2002) and Makino et al., (2003) have reported that a filamentous phage f237 integrates into the chromosome of \textit{V. parahaemolyticus}. The pattern of genomic organisation of phage f237 is similar to that of the CTXφ phage.

In conclusion, no clear distinction between clinical and environmental isolates of \textit{V. parahaemolyticus} could be made using plasmid analysis. Furthermore, the incidence of plasmids did not correlate with virulence of this bacterium. Future work could look at sequencing a number of clinical and environmental \textit{V. parahaemolyticus} strains derived plasmids and to look for any relationships between them.

\subsection*{4.4.7 Whole cell protein}

Fifty five \textit{V. parahaemolyticus} isolates (23 clinical and 32 environmental) were phenotypically characterised by comparing whole cell soluble proteins separated by SDS-PAGE.

The technique was not useful to differentiate between virulent and avirulent forms of \textit{V. parahaemolyticus}. The whole cell protein patterns of all \textit{V. parahaemolyticus} were similar; however, there were slight differences in the number of bands produced in some isolates but these differences were not related to virulence. Four environmental isolates (E12 and E13, E14, E15) produced extra band at 90 kDa, E12 was negative for all enzyme activities tested, while, the others were only positive for urease and protease, suggesting that this band not related with pathogenicity of these isolates. A band at 60 kDa has been found in only one clinical (C1) and one environmental (E16) isolates, C1 was positive for urease, protease and phospholipase, while E16 was positive for only urease and protease.
activity, there were some isolates that positive for at least three enzyme activities but did not have this band suggesting that this band probably not contributed to pathogenicity.

Standard SDS-PAGE used in the present study was not useful to differentiate isolates. 2D SDS-PAGE is used to separate complex mixtures of proteins. However, it is possible that 2D SDS-PAGE may offer means to differentiate between virulent and avirulent forms, but it is expensive and time consuming, especially with large number of bacteria to analyse the whole cell protein and ECPs. O’Farrell, (1975) has reported that 2D SDS-PAGE provides more information than 1D SDS-PAGE and is capable of resolving thousands of proteins in one procedure. 2D SDS-PAGE permits simultaneous determination of approximate isoelectric points of proteins by isoelectric focusing in the first dimension and molecular weights of proteins by sodium dodecyl sulphate electrophoresis in the second dimension. Kabir, (1991) has demonstrated that 2D SDS-PAGE has a high resolving power compared to that offered by 1D SDS-PAGE. More recently, 102 unique proteins were identified in Lactobacillus acidophilus NCFM by using the 2D SDS-PAGE based alkaline proteome (Majumder et al., 2012).

Bhoumick, et al., (2008) have used SDS-PAGE for evaluation of protein profiles for typing V. parahaemolyticus isolated from seafood and comparison with other techniques. The protein profiles revealed a difference in intraspecies at the protein level but the differences between isolates was not as distinct as that found from DNA fingerprinting; authors have reported that the differences between V. parahaemolyticus isolates using the protein profiling method were not distinct as molecular typing methods such as RAPD-PCR. Therefore, the authors
recommended using molecular typing technique to detect the differences between
the isolates.

4.4.8 ECPs profile

Many pathogenic *Vibrio* species are able to produce ECPs and these ECPs are
found to be related to pathogenicity (Elston and Leibovitz, 1980, Nottage and
Birkbeck, 1987). Maeda and Yamamoto, (1996) have reported that proteases are
mostly detected in these ECPs and cause host tissue damage. Moreover, ECPs
could also interfere with host defense factors. The ECPs of *V. parahaemolyticus*
may be associated with the pathogenicity of this bacterium (Cheng et al., 2008).

Comparative protein bands profile of ECPs was observed by SDS-PAGE. The
results show this bacterium produces few bands at 24 hours. However, *V.
parahaemolyticus* produces ECPs which were collected by harvesting a 2 day-old
culture and found to be somewhat variable among the isolates this results are
supported by Labreuche et al., (2006) who reported that ECPs protein bands
numerous and prominent and could be mainly produced in two days. The results
of the present study agree with Zhang and Austin, (2000) who have studied *V.
harveyi* and they found the ECPs protein profile revealed a very great variety
among the isolates of *V. harveyi*. Aguirre-Guzmán et al., (2004) have reported that
*Vibrio* species that were isolated from the environment secrete numerous ECPs as
virulence factors. These ECPs include haemolysin, protease and chitinase and
have a toxic effect on shrimp (Stensvag et al., 1993, Liu et al., 1997, Lee et al.,
1997).

In the present study, the ECPs that were harvested at 48 h produced more bands
than those from 24 h in some isolates. Thus it was necessary to note if those
ECPs were truly secreted or may be because of autolysis (lysis of the bacterial
cells and release of internal enzymes). A simple procedure was used to test the viability of bacterial cells in 24h and 48h by using trypan blue stain; the percentage of viability of *V. parahaemolyticus* cell was 77%, thus 23% nonviable cells in 24 h. A 66.5% for viable cells; nonviable cells were 33.5% in 48 h, suggesting that nonviable cell may produce bands detected by SDS-PAGE resulting from autolysis in some isolates but there is a good percentage 66.5% of viable cells.
CHAPTER 5

CYTOTOXIC EFFECT OF VIBRIO PARAHAEOMOLYTICUS EXTRACELLULAR PRODUCTS (ECPS) ON HUMAN COLORECTAL EPITHELIAL CELLS (Caco-2) AND CHINESE HAMSTER OVARY (CHO-K1) CELLS

Results from this Chapter have been presented at Centre for Research in Translational Biomedicine, Plymouth University, April 2011 and SGM Autumn Conference 2011, Global disease burden of enterics Display, York, September 2011. Results from this chapter have also been published in European Journal of Clinical Microbiology and Infectious Diseases 31:431–439 (Kadhim, et al., 2012)
Chapter 5: Cytotoxic Effect of *Vibrio parahaemolyticus* Extracellular Products (ECPs) on Human Colorectal Epithelial Cells (Caco-2) and Chinese Hamster Ovary (CHO-K1) Cells

5.1 Introduction:-

Studies have sought to ascertain the production both *in vitro* and *in vivo* of ECPs by various *Vibrio* species and their importance as potential virulence factors (Montero and Austin, 1999, Zorrilla et al., 2003). A large number of environmental *Vibrio* species are reported to produce extracellular toxic factors (Venkateswaran et al., 1991). Elston and Leibovitz, (1980) reported that there is a relationship between pathogenic *Vibrio* species isolated from oyster and their ability to produce ECPs. Aguirre-Guzmán et al., (2004) have demonstrated that a variety of *Vibrio* species isolated from the environment and marine organisms secrete several ECPs that have been demonstrated *in vivo* and *in vitro* as a main virulence factor for some fish pathogenic bacteria such as *V. damsela* (Fouz et al., 1993) and *V. alginolyticus* (Balebona et al., 1998). In the case of virulent *V. parahaemolyticus*, ECPs appear to play a role in the death of experimental animals, suggesting that this bacterium secretes one or more toxic factors (Liu et al., 2000). TDH is a membrane-active protein toxin free from carbohydrate and lipid (Honda and Iida, 1993) which causes *in vitro* haemolysis and cell death, but also causes morphological changes (e.g. loss of the number of microvilli on the cell surface, followed by loss of cytoplasmic substances), and causes the death of various cell lines (Sakurai et al., 1976, Tang et al., 1995). *In vivo*, cytotoxicity of TDH may play a role in destroying intestinal epithelial cells, leading to bloody mucous stool (Honda and Iida, 1993). Moreover, TDH causes intestinal fluid secretion (Raimondi et al., 2000). TDH may act as an indicator of potential virulence of *V. parahaemolyticus* correlating with human pathogenicity (Miyamoto et al., 1969).
Matsuda et al. (2010) have demonstrated that TDH induces cytotoxicity in cultured cells and it is a major virulence factor of this organism. The toxicity of TDH and TRH are similar. Although, most published studies on the cytotoxicity of *V. parahaemolyticus* isolates are related to TDH or TRH (Raimondi et al., 2000, Tang et al., 1995). Lynch et al., (2005) and Park et al., (2004b) have reported that this bacterium has cytotoxic activity even in the absence of TDH and TRH.

Elston (1980) demonstrated that ECPs have biological activities which may contribute to the disease development as aggressions enabling the bacteria to counteract the host defence systems. Lee et al., (2002a) have purified protease from supernatant of *V. parahaemolyticus* and found that protease caused *in vivo* haemorrhagic damage and death in mice after injection both intravenously and intraperitoneally.

The aim of this study was to develop a cytotoxicity assay using ECPs and measuring release of lactate dehydrogenes (LDH) as an indicator of cell lysis in two types of cell lines.
5.2. Materials and methods:-

5.2.1. Tissue culture and cell lines

The role of ECPs (section 4.2.8) in cytotoxicity of *V. parahaemolyticus* was determined in the human colorectal epithelial cells (Caco-2) and Hamster Chinese ovary (CHO-K1) cells.

Human colorectal epithelial cells (Caco-2) were grown in a 75-cm² flask and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, UK) (Appendix I) supplemented with 10% (v/v) fetal bovine serum (FBS), 5 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (to prevent bacterial contamination) and incubated in an atmosphere containing 5% CO₂ at 37°C for 21 days for cell confluence. The medium was replaced every 2–3 days to remove waste products and provide new nutrients. After confluent growth, the cells were washed twice with 0.53 mM Versene solution and treated with 0.25% trypsin-EDTA solution (for cell dissociation and to remove adherent cells from the culture surface). Before exposure to ECPs, cells were washed with DMEM without phenol red.

Chinese hamster ovary (CHO-K1) cells were cultured in 75-cm² flasks in Ham nutrient mixture F-12 supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, UK) at 37°C for 72 h. After confluent growth, cells were washed twice with phosphate-buffered saline (PBS) and treated with 0.25% trypsin-EDTA solution.

5.2.2. Assay for cytotoxic activity

The CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega, UK) was used to test cytotoxic activity of the ECPs of *V. parahaemolyticus* isolates by monitoring release of lactase dehydrogenase (LDH) according to the manufacturer’s instructions as follows.
5.2.2.1. Optimisation of target cell number

Because each target cell type has varying amounts of LDH, it was necessary to determine the optimum number of target Caco-2 and CHO-K1 cells to use in the assay.

Serial dilutions of Caco-2 and CHO-K1 cells (0, 5,000, 10,000, 20,000, 40,000, 80,000/100µl) were prepared in a V-bottom 96-well microtiter plates (Fisher Scientific International, Inc., UK), in triplicate. A triplicate set of wells for the culture medium background without cells was also prepared. In order to lyse cells, 15 µl of lysis solution (Appendix I) were added to all wells and then incubated in a humidified chamber for 45 min at 37°C and the plate was centrifuged at 500 x g for 5 min. From all wells, 50 µl aliquots were transferred to a fresh flat bottom 96-well (enzymatic assay) plate. The substrate preparation and LDH reaction were performed in the dark. Frozen Assay Buffer was melted in a 37°C water bath and 12 ml were added at room temperature to a bottle of Substrate Mix and gently shaken to dissolve the substrate. Next, 50 µl of the reconstituted Substrate Mix was added to each well of the plate and this was incubated for 30 min at room temperature. Finally, 50 µl of Stop Solution was added to each well. The absorbance at 490 nm was recorded using a Fusion Universal microplate analyser (Optimax, Molecular Devices UK Ltd.) after popping any large bubbles by using a syringe needle. The concentration of target cells was determined by yielding absorbance values at least two times the background absorbance of the medium control.
5.2.2.2 Cell-mediated cytotoxicity assay

(i) Assay plate setup

Approximately $5 \times 10^3$ trypsinised cells in 100 µl of the appropriate medium were dispensed into each well of the 96-well microtiter plates (Fisher Scientific International, Inc., UK), as determined in section (5.2.2.1).

A 15 µl of volume of ECPs from 23 clinical and 32 environmental isolates was added to each experimental well in triplicate. A triplicate set of “spontaneous” well; received 15 µl of sterile culture medium to ascertain release in the absence of ECPs. A triplicate set of maximum LDH release wells received 15 µl of lysis solution (10X) to yield complete lysis of target cells (as determined by phase contrast microscopy).

(ii) Cell culture and supernatant harvest

The cytotoxicity assay plate was incubated at 37°C for 4 hours to ensure sufficient contact between target and effector cells based on the manufacturer’s instructions. The cells in maximum LDH release wells were then treated with lysis solution for 45 min and the plate was centrifuged at 500 x g for 5 min. The substrate preparation and LDH reaction were performed as described in section (5.2.2.1).

(iii) LDH measurement

LDH measurement was performed as described in section (5.2.2.1), and determination of cytotoxicity calculations were based on the formula: % cytotoxicity = 100 x ([optical density at 490 nm ‘OD$_{490}$’ for experimental release ‘exp’ – OD$_{490}$ for spontaneous release ‘spon’]/ [OD$_{490}$ for maximum release ‘max’ – OD$_{490}$ for spontaneous release]), where the spontaneous release was the amount of LDH released from the cytoplasm of untreated cells, while the maximum release
was the amount of LDH release from toxin untreated cells that were totally lysed with the lysis buffer included in the kit.

(iv) Statistical analysis

Statistical analysis was performed using the appropriate parametric (student’s $t$-test) and non-parametric (Mann-Whitney $W$-test) by using Statgraphics Centurion XVI.I 16.01 (Plymouth University, UK). A $p < 0.05$ was considered statistically significant (Appendix II).
5.3. Results

The ECPs obtained from clinical and environmental isolates were tested for their cytotoxicity to both Caco-2 and CHO-K1 cells. The mean cytotoxicity value of clinical isolates was much greater than for environmental isolates (Figure 5.1). Clinical isolates showed 69.53% and 59.63% relative cytotoxicity in Caco-2 and CHO-K1 cells, respectively, while environmental isolates showed 17.48% and 12.18% relative cytotoxicity in Caco-2 and CHO-K1 cells, respectively. The difference between the means of clinical and environmental isolates was significant at a p value of < 0.05. With each cell line (Caco-2 and CHO-K1), each isolate was tested on three different occasions using three replicates.

The isolates were grouped according to their percentage cytotoxicity values; high (> 50%), medium (28-49%) and low (0-27%) categories. Amongst clinical isolates, 21 out of 23 (91%) showed high cytotoxicity, and 2 (8.69%) showed medium cytotoxicity. Amongst environmental isolates 2 out of 32 (6.25%) showed high cytotoxicity, 2 (6.25%) showed medium cytotoxicity and 28 (87.3%) showed low cytotoxicity on both Caco-2 and CHO-K1 cells (Figure 5.2).

Table 5.1 relates the virulence characteristic of all isolates tested (previously described in Chapter 4) to their cytotoxicity that has been investigated in the present chapter. For example, clinical isolate C15 was positive for all five enzyme activities tested and caused 87% and 66% cytotoxicity on Caco-2 and CHO-K1 cells respectively. Clinical isolate C1 was positive for only three enzyme activities (urease, protease and phospholipase) and caused 40% and 45% cytotoxicity on Caco-2 and CHO-K1 cells respectively. Likewise, clinical isolate C10 caused 42% and 47% cytotoxicity on Caco-2 and CHO-K1 cells respectively but was positive for urease, lipase and protease. However, cytotoxicity of ten environmental
isolates (E1, E4, E5, E6, E8, E10, E12, E17, E19 and E22) was less than 5% to both Caco-2 and CHO-K1; these isolates were negative for all enzymes tested and negative for both \textit{tdh} and \textit{trh} genes. Environmental reference strain NCTC (R2) was positive for urease, protease and phospholipase and caused 60.8% and 40% cytotoxicity on Caco-2 and CHO-K1 cells respectively.

For statistical analysis, the differences in cytotoxicity of ECPs of \textit{V. parahaemolyticus} isolates were compared: (i) KP$^+$ (β-haemolysis) versus KP$^-$ (ii) Phospholipase$^+$ versus phospholipase$^-$ isolates (iii) Protease$^+$ versus protease$^-$ isolates (iv) Urease$^+$ versus urease$^-$ (v) Lipase$^+$ versus lipase$^-$ isolates (vi) \textit{tdh}$^+$ versus \textit{tdh}$^-$ isolates (vii) \textit{trh}$^+$ versus \textit{trh}$^-$ isolates (Figures 5.3-4, Tables 5.2-3).

The mean cytotoxicity percentage of KP$^+$ \textit{V. parahaemolyticus} isolates was significantly higher ($p < 0.05$) than that for KP$^-$ isolates. KP$^+$ \textit{V. parahaemolyticus} isolates exhibited 64.76% and 53.73% relative cytotoxicity on Caco-2 and CHO-K1 cells respectively, whereas, KP$^-$ isolates exhibited 19.64% and 15.22% relative cytotoxicity on Caco-2 and CHO-K1 cells respectively. Similarly, cytotoxicity of phospholipase positive \textit{V. parahaemolyticus} isolates on both cell types was significantly higher ($p < 0.05$) than cytotoxicity of phospholipase negative strains.

Although there were significant differences ($p < 0.05$) between relative cytotoxicity of urease positive \textit{V. parahaemolyticus} isolates (51.77% and 41.62% on Caco-2 and CHO-K1 cells respectively) and urease negative of \textit{V. parahaemolyticus} isolates (11.27% and 11.11% on Caco-2 and CHO-K1 cells respectively) (see Figures 5.3-4 and Tables 5.2-3), there were some environmental isolates with low cytotoxicity, for example, E23 caused 14.8% and 5% on Caco-2 and CHO-K1 cells respectively Table 5.1. Likewise, there were also significant differences between cytotoxicity of protease positive \textit{V. parahaemolyticus} isolates and protease
negative isolates but there were some environmental isolates with low cytotoxicity, for example, E23 caused 14.8% on Caco-2 and 5% CHO-K1. The mean cytotoxicity percentage of lipase positive *V. parahaemolyticus* isolates was not significant.
Figure 5.1 Cytotoxic effects of ECPs of clinical and environmental isolates of *V. parahaemolyticus* on Caco-2 and CHO-K2 cell lines. Error bars represent the standard error of the mean.
Figure 5.2 Cytotoxicity (% relative to complete chemical cell lysis) of Caco-2 and CHO-K1 cell lines with ECPs of *Vibrio parahaemolyticus* isolates. Error bars represent the standard error of the mean.
Table 5.1 Virulence characteristics and cytotoxicity of *Vibrio parahaemolyticus* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type and source (where known)</th>
<th>Virulence Characteristics and virulence genes</th>
<th>Cytotoxicity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KP(β-haemolysis) Ure Lip Pro PPL tdh trh Caco-2 CHO-K1</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>Clinical, NCIMB Reference Strain, UK</td>
<td>+ - - + - - -</td>
<td>60.0 62.0</td>
</tr>
<tr>
<td>C1</td>
<td>Clinical, Norway</td>
<td>- + - + + -</td>
<td>40.0 45.0</td>
</tr>
<tr>
<td>C2</td>
<td>Clinical, Norway</td>
<td>+ + - + + +</td>
<td>85.0 71.9</td>
</tr>
<tr>
<td>C3</td>
<td>Clinical, Norway</td>
<td>- + - + - +</td>
<td>81.0 62.7</td>
</tr>
<tr>
<td>C4</td>
<td>Clinical, Norway</td>
<td>+ + + + + +</td>
<td>83.5 67.0</td>
</tr>
<tr>
<td>C5</td>
<td>Clinical, Norway</td>
<td>+ + + + + +</td>
<td>84.0 67.0</td>
</tr>
<tr>
<td>C6</td>
<td>Clinical, Norway</td>
<td>+ + + + + +</td>
<td>71.0 61.0</td>
</tr>
<tr>
<td>C7</td>
<td>Clinical, Norway</td>
<td>+ + + + + +</td>
<td>72.0 62.5</td>
</tr>
<tr>
<td>C8</td>
<td>Clinical, Norway</td>
<td>+ + + + + +</td>
<td>82.0 63.0</td>
</tr>
<tr>
<td>C9</td>
<td>Clinical, Norway</td>
<td>+ + + + + +</td>
<td>74.5 55.0</td>
</tr>
<tr>
<td>C10</td>
<td>Clinical, Norway</td>
<td>- + + + - -</td>
<td>42.0 47.0</td>
</tr>
<tr>
<td>C11</td>
<td>Clinical, Japan</td>
<td>+ + - - + -</td>
<td>83.6 66.6</td>
</tr>
<tr>
<td>C12</td>
<td>Clinical, Japan</td>
<td>+ + + + + -</td>
<td>83.7 66.0</td>
</tr>
<tr>
<td>C13</td>
<td>Clinical, Japan</td>
<td>+ + + + + +</td>
<td>65.0 54.5</td>
</tr>
<tr>
<td>C14</td>
<td>Clinical, Japan</td>
<td>+ + - - + -</td>
<td>65.0 58.0</td>
</tr>
<tr>
<td>C15</td>
<td>Clinical, UK</td>
<td>+ + + + + +</td>
<td>87.0 66.0</td>
</tr>
<tr>
<td>C16</td>
<td>Clinical, Spain</td>
<td>+ + + + + +</td>
<td>64.6 51.7</td>
</tr>
<tr>
<td>C17</td>
<td>Clinical, Spain</td>
<td>- + + + + -</td>
<td>65.0 52.0</td>
</tr>
<tr>
<td>C18</td>
<td>Clinical, Spain</td>
<td>+ + + + + +</td>
<td>61.9 61.0</td>
</tr>
<tr>
<td>C19</td>
<td>Clinical, Spain</td>
<td>+ + + + + +</td>
<td>64.0 64.0</td>
</tr>
<tr>
<td>C20</td>
<td>Clinical, Spain</td>
<td>+ + + + + +</td>
<td>60.0 60.5</td>
</tr>
<tr>
<td>C21</td>
<td>Clinical, Italy</td>
<td>+ + + + + +</td>
<td>55.8 52.0</td>
</tr>
<tr>
<td>C22</td>
<td>Clinical, UK</td>
<td>+ + + + + +</td>
<td>63.0 51.0</td>
</tr>
<tr>
<td>R2</td>
<td>Environmental, NCTC Reference Strain, UK</td>
<td>- + + + - -</td>
<td>60.8 40.0</td>
</tr>
<tr>
<td>E1</td>
<td>Environmental, Spain</td>
<td>- - - - - -</td>
<td>0.6 5.0</td>
</tr>
<tr>
<td>E2</td>
<td>Environmental, Spain</td>
<td>- - - - - -</td>
<td>19.8 4.7</td>
</tr>
<tr>
<td>E3</td>
<td>Environmental, Spain</td>
<td>- - - - - -</td>
<td>39.0 37.5</td>
</tr>
<tr>
<td>E4</td>
<td>Environmental, Spain</td>
<td>- - - - - -</td>
<td>0.5 4.9</td>
</tr>
<tr>
<td>E5</td>
<td>Environmental, Spain</td>
<td>- - - - - -</td>
<td>0.6 3.0</td>
</tr>
<tr>
<td>E6</td>
<td>Environmental, Spain</td>
<td>- - - - - -</td>
<td>0.5 4.0</td>
</tr>
<tr>
<td>E7</td>
<td>Environmental, UK</td>
<td>+ - - + + +</td>
<td>20.5 19.5</td>
</tr>
<tr>
<td>E8</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>0.4 5.0</td>
</tr>
<tr>
<td>E9</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>86.6 62.0</td>
</tr>
<tr>
<td>E10</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>0.6 3.0</td>
</tr>
<tr>
<td>E11</td>
<td>Environmental, UK</td>
<td>+ - - - - -</td>
<td>19.0 3.0</td>
</tr>
<tr>
<td>E12</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>0.6 3.0</td>
</tr>
<tr>
<td>E13</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>18.0 5.7</td>
</tr>
<tr>
<td>E14</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>19.6 4.0</td>
</tr>
<tr>
<td>E15</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>20.0 9.8</td>
</tr>
<tr>
<td>E16</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>25.9 25.8</td>
</tr>
<tr>
<td>E17</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>0.4 4.8</td>
</tr>
<tr>
<td>E18</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>24.5 11.0</td>
</tr>
<tr>
<td>E19</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>0.4 4.0</td>
</tr>
<tr>
<td>E20</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>28.0 29.5</td>
</tr>
<tr>
<td>E21</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>0.5 5.0</td>
</tr>
<tr>
<td>E22</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>0.1 4.0</td>
</tr>
<tr>
<td>E23</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>14.8 5.0</td>
</tr>
<tr>
<td>E24</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>22.0 13.7</td>
</tr>
<tr>
<td>E25</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>0.6 4.5</td>
</tr>
<tr>
<td>E26</td>
<td>Environmental, UK</td>
<td>- - - - - -</td>
<td>26.7 12.0</td>
</tr>
<tr>
<td>E27</td>
<td>Environmental, Portugal</td>
<td>+ - - - + +</td>
<td>19.0 5.0</td>
</tr>
<tr>
<td>E28</td>
<td>Environmental, Portugal</td>
<td>+ - - - + +</td>
<td>27.0 20.0</td>
</tr>
<tr>
<td>E29</td>
<td>Environmental, Portugal</td>
<td>+ - - - + +</td>
<td>16.5 5.0</td>
</tr>
<tr>
<td>E30</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>24.7 15.8</td>
</tr>
<tr>
<td>E31</td>
<td>Environmental, UK</td>
<td>+ - - - + +</td>
<td>18.5 5.8</td>
</tr>
</tbody>
</table>
Figure 5.3 Cytotoxicity of ECPs of *V. parahaemolyticus* on Caco-2 cells depended on phenotypes (enzyme activity) and genotypes (known virulence gene). Error bars represent the standard error of the mean. Mean cytotoxicity percentages are noted in table 5.2. An asterisk indicates that the cytotoxicity difference between each group is significantly different (*p* < 0.05).
Figure 5.4 Cytotoxicity of ECPs of *V. parahaemolyticus* on CHO-K1 cells depended on phenotypes (enzyme activity) and genotypes (known virulence genes). Error bars represent the standard error of the mean. Mean cytotoxicity percentages are noted in table 5.3. An asterisk indicates that the cytotoxicity difference between each group is significantly different (*p* < 0.05).
Table 5.2 Cytotoxicity of ECPs of *V. parahaemolyticus* on Caco-2 cell line depended on virulence characteristics.

<table>
<thead>
<tr>
<th>Virulence characteristics</th>
<th>Mean cytotoxicity percentages of <em>V. parahaemolyticus</em> isolates</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive reactions</td>
<td>negative reactions</td>
<td></td>
</tr>
<tr>
<td>KP (β-haemolysis)</td>
<td>64.76 *</td>
<td>19.64 *</td>
<td></td>
</tr>
<tr>
<td>Phospholipase</td>
<td>66.86 *</td>
<td>17.87 *</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td>50.66 *</td>
<td>21.41 *</td>
<td></td>
</tr>
<tr>
<td>Urease</td>
<td>51.77 *</td>
<td>11.27 *</td>
<td></td>
</tr>
<tr>
<td>Lipase</td>
<td>47.65</td>
<td>37.15</td>
<td></td>
</tr>
<tr>
<td><em>tdh</em></td>
<td>62.35 *</td>
<td>22.65 *</td>
<td></td>
</tr>
<tr>
<td><em>trh</em></td>
<td>47.30</td>
<td>36.76</td>
<td></td>
</tr>
</tbody>
</table>

* An asterisk indicates that the cytotoxicity difference between each group is significantly different (*p* < 0.05).

Table 5.3 Cytotoxicity of ECPs of *V. parahaemolyticus* on CHO-K1 cell line depended on virulence characteristics.

<table>
<thead>
<tr>
<th>Virulence characteristics</th>
<th>Mean cytotoxicity percentages of <em>V. parahaemolyticus</em> isolates</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive reactions</td>
<td>negative reactions</td>
<td></td>
</tr>
<tr>
<td>KP (β-haemolysis)</td>
<td>53.73 *</td>
<td>15.22 *</td>
<td></td>
</tr>
<tr>
<td>Phospholipase</td>
<td>55.83 *</td>
<td>13.59 *</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td>40.81 *</td>
<td>18.83 *</td>
<td></td>
</tr>
<tr>
<td>Urease</td>
<td>41.62 *</td>
<td>11.11 *</td>
<td></td>
</tr>
<tr>
<td>Lipase</td>
<td>39.23</td>
<td>30.22</td>
<td></td>
</tr>
<tr>
<td><em>tdh</em></td>
<td>51.08 *</td>
<td>18.32 *</td>
<td></td>
</tr>
<tr>
<td><em>trh</em></td>
<td>36.51</td>
<td>30.63</td>
<td></td>
</tr>
</tbody>
</table>

* An asterisk indicates that the cytotoxicity difference between each group is significantly different (*p* < 0.05).
5.4 Discussion

It was necessary to determine the cytotoxicity characteristics of ECPs of *V. parahaemolyticus* isolated from clinical and environmental samples to understand their possible pathogenic mechanisms. Cytotoxicity of ECPs to Caco-2 and CHO-K1 cells was performed to assess the potential virulence of both clinical and environmental isolates of *V. parahaemolyticus* using quantity of LDH released. The isolates that show high cytotoxicity values are likely to be highly virulence, and the isolates that show medium and low cytotoxicity are medium and low virulence respectively. For the purposes of this work those isolates with more than 50% cytotoxicity were regarded as highly virulent strains and those with 28-49% were regarded as virulent strains but less virulence and those with less than 28% low virulence or avirulent.

In general, the clinical isolates showed a higher level of cytotoxicity than the environmental isolates. A minority of environment isolates showed similar levels of cytotoxicity to the proven virulent clinical isolates and these environmental isolates may be potentially virulent, for example, E9 caused high cytotoxicity because this isolate has the major virulence factor *tdh* as well as *trh*, plus putative virulence factors KP haemolysis and phospholipase. Very similar, if not identical, results were obtained with the same set of isolates whose ECPs were tested for cytotoxicity against Caco-2 and CHO-K1 cell lines. As a group, clinical isolates were more cytotoxic than environmental isolates. These results agree with Yeung et al., (2007) who used cytotoxicity assay to differentiate between pathogenic and non-pathogenic *V. parahaemolyticus* strains by using a tissue culture-based assay.
The enzyme activities previously determined (Chapter 4) were compared with the cytotoxicity of ECPs in clinical and environmental *V. parahaemolyticus* isolates.

Statistical analysis showed that for *V. parahaemolyticus* isolates urease, protease, phospholipase and Kanagawa haemolysis activities and possession of *tdh* positive reaction were significantly related to cytotoxicity to Caco-2 and CHO-K1 cells. The presence of the *trh* gene in either clinical (6/23) or environmental (7/32) isolates was low; therefore, mean cytotoxicity percentage of *trh* positive strains was not significant. Vongxay et al., (2008) have detected the *trh* gene in 3 of 25 clinical and 7 of 191 environmental isolates and suggested that TRH and TDH are not the only virulence factors responsible for *V. parahaemolyticus* pathogenicity. Wong et al., (1992) have reported that the KP+ *V. parahaemolyticus* has been involved in gastroenteritis. However, some strains of KP− *V. parahaemolyticus* are involved in bacteraemia (Charlton et al., 1999). In the present study, some isolates lacked *tdh* and *trh* but showed cytotoxicity supporting the results of García et al., (2009) who reported that *V. parahaemolyticus* retained pathogenicity in the absence of *tdh* and *trh* genes. Moreover, pathogenic *V. parahaemolyticus* lacking *tdh* and *trh* genes, still demonstrated cytotoxic activity and death in a mouse model (Xu et al., 1994).

The present study demonstrated that Kanagawa reaction and phospholipase gave similar but not identical correlations with cytotoxicity. For example, most *V. parahaemolyticus* isolates that were positive for KP also were positive for phospholipase but C1, C3, R2 and E3 were negative for Kanagawa reaction and positive for phospholipase and they were still cytotoxic (Table 5.1), suggesting that phospholipase may contribute to pathogenicity of these isolates tested. Phospholipases hydrolyse phospholipids that are present in the host cell membrane, contributing to membrane damage (Schmiel et al., 1998, Istivan and
Coloe, 2006); in other bacteria, phospholipases also play a role in nutrient digestion in the formation of bioactive molecules. For example, phospholipase in *Clostridium perfringens* (alpha-toxin) causes massive tissue destruction such as gas gangrene; and *Listeria monocytogenes* produces a phospholipase involved in food borne infection (Titball, 1993, Songer, 1997).

In the present study, the mean cytotoxicity percentage exhibited by lipase positive *V. parahaemolyticus* isolates was similar to lipase negative isolates, suggesting that lipase does not contribute to the pathogenicity of *V. parahaemolyticus*. The cytotoxicity of protease positive *V. parahaemolyticus* isolates was significantly higher than protease negative isolates. However, Wong et al., (1992) have demonstrated that lipases and proteases of *Vibrio* species have a role in cytotoxicity of CHO-K1 cells. Lin and Lee, (1997) have reported that protease activity caused cytotoxicity to CHO-K1 cells. Furthermore, Sudheesh and Xu, (2001) have injected prawns with ECPs, containing protease, from a potentially pathogenic environmental *V. parahaemolyticus* strain and this caused mortality of all the prawns injected. The ECPs produced by many bacterial pathogens include proteases as virulence factors which lyse host cells (Lantz, 1997). Labreuche et al., (2006) have studied toxicity to oysters of ECPs from pathogenic *V. aestuarianus*. Most bacterial proteases can cause extensive host tissue disruption facilitating the propagation of the bacteria. Thus, proteases degrade host proteins to provide nutrients for growth of the bacteria (Maeda and Yamamoto, 1996).

In the current study, urease enzyme activity was considered as a marker for cytotoxicity, but is unlikely to be responsible for cytotoxicity, although there was a significant difference between the mean cytotoxicity percentage of urease positive and negative *V. parahaemolyticus* isolates. Depaola et al., (2000) have reported
that urease positive *V. parahaemolyticus* strains and possession of *trh* and *tdh* genes did not enhance the virulence of those strains. However, Park, (2004c) has demonstrated that environmental urease positive *V. parahaemolyticus* strains caused mice mortality when injected, and these strains were recovered from viscera of injected mice, and they showed much higher mortality than haemolytic and protease positive strains (Park, 2004d).

Importantly, it may be possible that collectively these enzyme activities have a role in cytotoxicity. In other words, there are at least two or three or more possible enzyme factors responsible for cytotoxicity. However, lack of one or more of these components may still leave the strain virulent.

This chapter primarily verified the virulence of clinical isolates by analysing putative virulence factors which could possibly be used to differentiate between potentially pathogenic and non-pathogenic forms of *V. parahaemolyticus*. It clearly differentiates between virulent and avirulent isolates. However, this is not suitable for rapid identification of *V. parahaemolyticus* in foodstuffs as there is the need to isolate, grow the organism in broth and use the concentrated supernatant for cytotoxicity testing. There is no possibility for using this assay directly with foodstuffs without isolation due to the interference from other agents, both microbial and nutritional in the food.

A different procedure has been developed by Yeung et al., (2007) to detect the presence of pathogenic *V. parahaemolyticus* directly from seafood using artificially inoculated (spiked) oyster homogenates with approximately $5 \times 10^8$ CFU of FSL-Y1-024 culture. The authors have reported that this assay can be used to detect the presence of more than $10^5$ cells of a pathogenic *V. parahaemolyticus* strain in an oyster matrix.
Similar assay (cytotoxicity by measuring the amount of lactate dehydrogenase released from different cell lines by the CytoTox96® Non-Radioactive Cytotoxicity Assay kit) can be used to test the cytotoxic activity for other bacteria, for example, this assay has been used to test the cytotoxic activity of *V. vulnificus* (Kim et al., 2003b) and to test the cytotoxic effect of phospholipase that produced from *V. mimicus* using fish cell line (Lee et al., 2002b).
CHAPTER 6

RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD)-PCR AND DEVELOPMENT OF A MULTIPLEX POLYMERASE CHAIN REACTION (M-PCR) FOR DETECTION AND IDENTIFICATION OF VIRULENT AND AVIRULENT FORMS OF VIBRIO PARAHAEOMOLYTICUS

Results from this Chapter have been presented at Vibrio 2010, Biloxi, Mississippi, USA, November 2010, and SGM Autumn Conference 2011, Global disease burden of enterics Display, York, September 2011, Vibrio 2011, Santiago de Compostela, Spain, November 2011, and Centre for Research in Translational Biomedicine (CRTB), Plymouth University, July 2012. The results from this chapter have also been published in European Journal of Clinical Microbiology and Infectious Diseases 31:431–439 (Kadhim, et al., 2012).
Chapter 6: Randomly Amplified Polymorphic DNA (RAPD)-PCR and Development of a Multiplex Polymerase Chain Reaction (M-PCR) for Detection and Identification of Virulent and Avirulent Forms of *Vibrio parahaemolyticus*

6.1 Introduction

The distinction between virulent and avirulent strains of a species may require the use of molecular typing methods; for example, randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) is a technique that may be used to produce a unique DNA band pattern to differentiate virulent strains from avirulent strains. It has been used by Warner and Oliver (1999) to distinguish virulent forms of *V. vulnificus* isolates by producing a unique DNA band profile in clinical isolates, and it is suggested that such DNA may play a role in the virulence of this bacterium. Furthermore, RAPD fingerprinting was developed as a useful tool in distinguishing the pathogenic and non-pathogenic isolates of *V. harveyi* (Hernández and Olmos, 2004, Alavandi et al., 2006).

RAPD uses primers under low specificity conditions to produce PCR amplicon (Williams et al., 1990, Welsh and Mcclelland, 1990). RAPD-PCR can generate various fingerprint profiles with very little template DNA and an unlimited number of primers (Leal et al., 2004). The primers used are simple, short and randomly chosen without prior knowledge of the template DNA sequence (Blixt et al., 2003). Arbitrary DNA sequences are used as single primers which target unspecified genomic sequences to generate a genetic profile, therefore it does not require any sequence data (Caetano-Anolles et al., 1991). Hu et al., (1995) used two primers in the RAPD reaction and found that the two-primer RAPD tends to produce more
bands and of a smaller size than the standard RAPD technique using only one primer.

There are simple, validated molecular methods for identification of the species *V. parahaemolyticus*. A toxR targeted by PCR for specific detection of *V. parahaemolyticus* producing a 368 bp amplicon has been developed by Kim et al., (1999). Okura et al., (2003) developed a new M-PCR targeting either *orf8* or *toxRS* or both simultaneously, to detect the virulent strains of *V. parahaemolyticus*. Furthermore, Tarr et al., (2007) have used M-PCR by amplifying *rpoB* gene to identify four *Vibrio* species yielded the species-specific amplicon to produce product size 897 bp for *V. parahaemolyticus*, 248 bp for *V. cholerae*, 121 bp for *V. mimicus* and 410 bp for *V. vulnificus* in a single reaction. Another M-PCR which targets the gene coding for collagenase and specifically identifies three *Vibrio* species (*V. parahaemolyticus*, *V. alginolyticus* and *V. cholerae* O1 and O139) has been developed by Di Pinto et al., (2005). Moreover, Castroverde et al., (2006) have demonstrated a M-PCR to detect three types of *Vibrio* shrimp pathogens. Bej et al., (1999) have developed a M-PCR to target three genes, the species specific marker *tlh* gene to identify *V. parahaemolyticus* and pathogenicity marker *tdh* and *trh* (haemolysin) genes to detect the virulent forms of *V. parahaemolyticus* in the same tube of PCR reaction.

This chapter describes a rapid and simple method for differentiating virulent clinical and mainly avirulent environmental isolates of *V. parahaemolyticus*, which led in turn to the development a M-PCR that can be used to reliably identify all isolates of the *V. parahaemolyticus* species tested and detect potentially virulent strains, simultaneously. To achieve this, a RAPD method was used with novel sets of arbitrary primers to identify unique RAPD bands in known virulent isolates and
their sequence information was used to develop the M-PCR-based method. The M-PCR method presented here combines the OMP-PCR and HTP-PCR with a ToxR-PCR allowing the identification of potentially virulent forms of *V. parahaemolyticus*. 
6.2 Materials and methods

6.2.1 RAPD-PCR of *V. parahaemolyticus*

RAPD analysis was performed using the DNA obtained from 55 isolates of *V. parahaemolyticus* and RAPD Analysis Beads (Amersham Biosciences) and a set of P1 (5'-d [GGTGCGGGAA]-3’) and P5 (5'-d [AACGCGCAAC]-3’) 10-mer primers. For each reaction, a bead was resuspended in a 25 µl volume containing 1 unit Amplitaq DNA polymerase and Stoffel fragment, 0.4 mM of each dNTP, 2.5 µg BSA and buffer [3 mM MgCl₂, 30 mM KCl and 10 mM Tris, (pH 8.3)]. For each reaction 25 pmol (5µl) of the appropriate primer and DNA template (10 ng) were added to generate optimum banding patterns for agarose gel electrophoresis. All RAPD-PCR reactions were performed in a Primus 96 plus thermal cycler (MWG Biotech) programmed for one cycle consisting of: 95°C for 5 min and 45 cycles consisting of 95°C for 1 min, 36°C for 1 min and 72°C for 2 min.

6.2.2 Screening of RAPD primers using OPK singly and in pairs.

RAPD-PCR was performed as above but with RAPD primers OPK. Genomic DNA from a panel of 4 isolates of *V. parahaemolyticus* was screened with 9 randomly chosen 10-mer oligonucleotide single primers (OPK1-7, OPK12 and OPK18) and 30 primer pairs obtained from RAPD 10-mer kits (Eurofins MWG Operon, UK) (Table 6.1), and used to screen for unique banding patterns. The combination of OPK primers (30 reactions) used is shown in Figure 6.8.
Table 6.1 The sequences of OPK primers used in the RAPD-PCR

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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>%GC</th>
</tr>
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<td>60%</td>
</tr>
<tr>
<td>OPK-02</td>
<td>5'GTCTCCGCAA-3'</td>
<td>60%</td>
</tr>
<tr>
<td>OPK-03</td>
<td>5'CCAGCTTAGG-3'</td>
<td>60%</td>
</tr>
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<td>OPK-04</td>
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</tr>
<tr>
<td>OPK-06</td>
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<td>OPK-07</td>
<td>5'AGCGAGCAAG-3'</td>
<td>60%</td>
</tr>
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<td>60%</td>
</tr>
<tr>
<td>OPK-10</td>
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<tr>
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<tr>
<td>OPK-12</td>
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<td>OPK-18</td>
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6.2.3 PCR-OMP

PCR primers to detect the outer membrane protein (OMP) sequence of virulent isolates were constructed. VPOMP1 and VPOMP2 were designed as described in section 2.5 and PCR was performed as described in section 2.6. PCR conditions started with 1 cycle of pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60°C for 1 min, and elongation at 70°C for 2 min. The amplification ended with a final elongation at 70 °C for 5 min. Next, 10 µl of amplified PCR products were electrophoresed in 2 % agarose gels submerged in 1x TBE buffer (pH 8), with 100 bp DNA ladder (Invitrogen, UK). Gel electrophoresis was performed as described in section 2.9.

6.2.4 PCR-HTP

PCR primers VPHTP1 and VPHTP2 were designed as described in section 2.5. The PCR was performed as described in section 2.6. PCR conditions were as follows: one cycle consisting of 94°C for 5 min, 30 cycles consisting of 94°C for 1 min, 59°C for 1 min, and 70°C for 2 min, and a final cycle consisting of 70°C for 5 min. The PCR products were electrophoresed as described in section 2.9.

6.2.5 Cloning and sequencing of DNA fragment

PCR fragments were excised and extracted from electrophoresis gel by using an agarose gel extraction kit (Roche Applied Science, UK) as described in section 2.7 and the amplified PCR product was purified by using the ‘High Pure PCR Product Purification Kit’ (Roche Applied Science, UK) according to the manufacturers’ instruction as described in section 2.8 and ligated into pGEM-T Easy vector (Promega, UK). The ligation product was transformed into E. coli JM109 as described in section 2.10.1-2 and cultured on LB/ampicillin/X-Gal/IPTG as described in section 2.10.3. Next, recombinant plasmid DNA was isolated from.
white colonies showing inserts using the Miniprep Plasmid DNA Extraction Kit (Bio-Rad, Hemel Hempstead, UK) as described in section 2.10.4 and cloning was verified by digestion with restriction enzyme EcoR1 to ensure that the plasmid contained the DNA fragment of interest, as described in section 2.10.5. The cloned DNA was sequenced by MWG-Biotech Ltd., Germany. Computer analysis of the DNA sequence data was performed via the National Centre for Biotechnology Information (NCBI) using GenBank databases search and BLAST programs (NCBI). The nucleotide sequences were submitted to GenBank (accession numbers JX401366 and JX401367).

6.2.6 Sequences of PCR products

Both strands of 200 bp purified PCR-OMP product and 300 bp purified PCR-HTP were sequenced by MWG Biotech. For confirmatory purposes, amplicons derived from three isolates were sequenced. These sequences, together with the nucleotide sequences of *V. parahaemolyticus* RIMD 2210633 were aligned using the online programs using software Multalin version 5.4.1 http://www-archbac.u-psud.fr/genomics/multalin.html (Corpet, 1988) sequences were also compared with those from other organisms in the GenBank database using the BLAST program at http://blast.ncbi.nlm.nih.gov (Altschul et al., 1990).

6.2.7 DIG-DNA labelling probe

The PCR-OMP product from the genome of reference strain R1 was used to develop a digoxigenin (DIG) labelled probe. Details of all the reagents used in this study can be found in Appendix I. The random primed method of labelling of DNA probe was performed according to the Roche instruction manual ‘DIG DNA Labelling and Detection Kit’. The PCR product was purified using the ‘High Pure PCR Product Purification Kit’ and DNA was quantified using the method described.
in section 2.4. The purified PCR product (0.5-2 µg) was added to molecular grade water to a final volume of 15 µl and denatured by boiling in a water bath for 10 min and rapidly cooling on ice. Next 2 µl of Hexanucleotide Mixture (10x), 2 µl of dNTP Labelling Mixture and 1 µl of Klenow enzyme were added. The tube contents were vortexed, centrifuged briefly and incubated at 37°C for approximately 3 h, after which the reaction was stopped by adding 2 µl of 0.2 M EDTA solution.

6.2.8 Preparation of dot-blot
A 5 µl volume containing 200 ng (40 ng/ µl) of genomic DNA was denatured by boiling in a water bath for 10 min and kept on ice immediately. Denatured DNA was spotted onto a nylon membrane. After drying the membrane, the membrane was placed on Whatman 3 MM- paper soaked with 10 x SSC. The spotted DNA was fixed to the membrane by UV-crosslinking (Chromato-Vue cabinet, Module: CC-60 UVP Inc) for 5 min. Membrane was rinsed briefly in molecular grade water and air dried.

6.2.9 DNA-DNA Hybridisation
A 20 ml volume of preheated DIG Easy Hyb solution (Roche) (65 °C) was used for a prehybridisation wash of the blot in a roller bottle for 30 min at 65 °C with gentle agitation using a Hybridiser HB-1D (Techne, Ltd, UK). DIG labelled DNA probe was denatured by boiling for 5 min and immediately cooling on ice. Denatured DIG- labelled DNA probe was added to a preheated (65 °C) 5 ml volume of DIG Easy Hyb solution at a final concentration of about 25 ng/ ml and mixed well. Prehybridisation solution was discarded to be replacing with the probe/DIG Easy Hyb mixture and incubated overnight in the roller bottles at 65 °C with gentle agitation using the Hybridiser. Post hybridisation stringency washes were performed twice in 20 ml of 2x SSC containing 0.1% SDS for 5 min at RT followed
by two further washes in 10 ml of prewarmed 0.5x SSC containing 0.1 SDS for 15 min at 65°C.

**6.2.10 Immunological detection**

After stringency washes, the membrane was rinsed in Washing Buffer (Appendix I) for 2 min, and then incubated in 100 ml of Blocking Solution (Appendix I) for 30 min (for blocking of unspecific binding sites on the membrane). The membrane was placed in a solution containing anti-digoxigenin antibody conjugated to alkaline phosphatase diluted 1:5000 in Blocking solution for 2 h at RT (for binding to the DIG-labelled probe), followed by washing the membrane twice in 100 ml of Washing Buffer for 15 min to remove any unbound antibody. The membrane was equilibrated in 20 ml Detection Buffer for 3 min, followed by incubation in 10 ml of Colour Substrate solution (for visualisation of antibody-binding) in the dark until a coloured precipitate formed. The reaction was stopped by washing the membrane with sterile molecular grade water. An image of the developed membrane was captured onto a digital camera.

**6.2.11 Multiplex PCR amplifications**

Multiplex PCR amplification was performed on genomic DNA from each of the isolates of *V. parahaemolyticus*, other *Vibrio* species and non-*Vibrio* species by the simultaneous addition of three primer pairs in a 50 µl reaction mixture consisting of 30 pmol of each of the oligonucleotide primers, 0.2 mM of each deoxynucleoside triphosphate, 1x reaction buffer (Appendix I), 60 ng of DNA template, 2 U of *Taq* DNA Polymerase (Roche) and the reaction volume was made up to 50 µl using PCR grade water. The primer pairs used in this PCR were as follows:
PCR conditions were optimised for an initial denaturation of 3 min at 94°C followed by 30 cycles each having denaturation for 1 min at 94°C, annealing for 1 min at 59°C, and extension for 2 min at 72°C, and a final extension step for 5 min at 72°C. The verification of the M-PCR products was performed by agarose gel electrophoresis as described in section 2.9.
6.3 Results

6.3.1 RAPD-PCR using P1 and P5 primers

This study builds upon the initial observation by Miah (2009) that RAPD performed with Amersham P1 and P5 primer set produces a unique band in some isolates of *V. parahaemolyticus*. This band was named band Y. In the present study, band Y was commonly observed as an approximately 600 bp fragment on electrophoresis of PCR products produced from clinical isolates and rarely from environmental isolates tested (Figure 6.1).

The 600 bp amplified DNA fragment was purified from agarose gels and cloned into the vector pGEM using *E. coli* JM109 as the host organism and then plasmid DNA was extracted, after blue/white screening, from white colonies. The restriction enzyme *EcoR1* and gel electrophoresis was used to verify cloning of the fragment (Figure 6.2). Derived sequences of the fragment are shown in Figure 6.3. BLAST analysis in comparison with sequences available at GenBank and EMBL (Altschul et al., 1990), revealed that the 600 bp sequence of the obtained RAPD-PCR fragment was identical to the sequences occupying 984020 bp to 984595 bp on chromosome 1 of *V. parahaemolyticus* RIMD 2210633 DNA (accession no.BA000031.2). The nucleotide sequence characterised as a gene encoding a putative outer membrane protein, with 100% homology for *V. parahaemolyticus*. At the protein level, OMP was similar to a putative outer membrane protein from *V. alginolyticus* (accession no. ZP_01260526.1, 93 % homology), and a conserved hypothetical protein of *V. harveyi* (accession no. ZP_06175389.1, 90 % homology).

Based on the DNA sequence of gene *omp*, the specific primers VPOMP1 (5'-GTCACGCGGCCAAACAAAGAGA-3') and VPOMP2 (5'-ACCGCATATCCTTGTTGGCTGGG-3') were developed. Using this primer pair, PCR was performed on genomic DNAs of a collection of 55 *V. parahaemolyticus*
isolates, together with 22 isolates of various other *Vibrio* species and five isolates of non-*Vibrio*. The expected 200 bp product was found in the 21 out of 23 clinical isolates of *V. parahaemolyticus*, including NCIMB reference strain R1 from clinical source and only 1 out of 32 environmental isolates. No band was seen with any of the other *Vibrio* and non-*Vibrio* species tested (Figure 6.4).

The nucleotide sequence analysis confirmed the identity of PCR products. Multiple sequence alignments from three isolates have been analysed. The sequences of the three isolates showed two single nucleotide polymorphisms (SNPs) appeared at the third base of their codon. The SNPs do not translate into a different amino acid (Figure 6.5).

The presence of the *omp* sequence was confirmed by DNA hybridisation. DIG labelled probe that developed from the specific PCR product of reference strain R1 was hybridised successfully to most of the DNA samples from clinical forms and only to a few environmental isolates (Figure 6.6). The hybridisation results were compared with results of PCR, in all cases confirmed the PCR product results (Table 6.2). Very weak signals in dot blot analysis (even after repeat testing) were obtained with four environmental (E24, E30, E18 and E28) isolates that did not show any OMP-PCR amplicon. Additionally, one clinical isolate (C1) and three environmental (E20, E16 and E3) isolates that were shown to be positive by dot blot analysis but by PCR appeared negative. Other species yielded negative results (no hybridisation signals).
6.3.2 RAPD-PCR using OPK primers

Initially, *V. parahaemolyticus* genomic DNA from two highly cytotoxic clinical isolates (C8 and C15) and two low cytotoxicity environmental isolates (E2 and E22) were screened with nine single primers OPK1-7, OPK12 and OPK18 in RAPD-PCR reactions. These primers failed to produce unique bands to differentiate the test clinical and environmental isolates of *V. parahaemolyticus* (Figure 6.7). However, when 30 different OPK primer pairs were used with the same selected isolates, one pair OPK13 +15 produced a unique band with the clinical isolates (Figure 6.8). This amplicon was named band Z. When the OPK13+15 primer set was used on the complete collection of *V. parahaemolyticus* isolates, it was found that a unique band of approx. 310 bp was produced by the majority of clinical isolates, but not by the vast majority of environmental forms (Figure 6.9). The commercial kit of standardised Ready to Go beads were used to avoid pipetting steps and all ingredients for reactions, guaranteeing equal amounts and concentration. Patterns generated by this RAPD-PCR were reproducible in assays on different occasions; a comparison of repeat RAPD-PCR showed that band Z (indicated by arrows in Figure 6.10) was present in all repeated experiments. However, the band was present with variable intensity.

The DNA from band Z was isolated and ligated into pGEM-T Easy vector and transformed into *E. coli* JM109. After blue/white screening, recombinant plasmid DNA was extracted from selected white colonies. Restriction enzyme analysis was used to verify the presence of expected size inserts within the clones (Figure 6.11) and selected recombinant plasmids were sequenced. BLAST analysis and comparison with the genome of *V. parahaemolyticus* RIMD 2210633 DNA (accession no.BA000031.2) showed identity with a 312 bp segment from base pairs 1812453 to 1812764 on chromosome 1. The nucleotide sequence was
analysed and found to be from a gene encoding a hypothetical protein (*htp*). The sequence of the 312 bp band Z was identical to the *htp* gene from *V. parahaemolyticus*. At the protein level, HTP was similar to proteins from *V. alginolyticus* (accession no. ZP_01259705.1, 62% homology), and *V. harveyi* (accession no. YP_001444888.1, 39% homology).

Based on the DNA sequence of gene *htp* the specific primers VPHTP1 (5'-'GACATCCATCTGCACGCAAC-3') and VPHTP2 (5'-'CCTTTCGCTTCGAGCAATCA-3') were developed (Figure 6.12). PCR was performed using this primer pair on genomic DNA of *V. parahaemolyticus* isolates, other *Vibrio* species and non-*Vibrio* species. The expected 300 bp product was produced by 19 out of 23 (82.6%) clinical isolates of *V. parahaemolyticus*, including clinical NCIMB reference strain R1 and only 2 out of 32 (6.25 %) environmental isolates (Figure 6.13). No band was seen with any of the other *Vibrio* and non-*Vibrio* species tested.

The nucleotide sequence analysis confirmed the identity of PCR products. Multiple sequence alignments from three isolates have been analysis. The sequences of the three isolates showed five single nucleotide polymorphisms (SNPs) appearing at the third base of their codon. The SNPs do not translate into a different amino acid (Figure 6.14).

In support of the PCR results, dot blot hybridisation confirmed the presence or absence of *htp*. All isolates that were positive in PCR were positive in dot blot analysis (Table 6.2). An additional two clinical (C6 and C9) and four environmental isolates (E28, E7, E9 and E16) were identified as possessing a *htp* gene sequence by dot blot hybridisation but not by PCR. Other species gave negative signals in dot blot analysis (Figure 6.15).
6.3.3 Multiplex PCR

This M-PCR reliably identified all *V. parahaemolyticus* isolates tested and distinguished between known virulent and avirulent strains simultaneously (Figure 6.16). The amplification of the species-specific toxR gene 367 bp segment was found in all *V. parahaemolyticus* isolates tested here. Out of 23 clinical isolates tested, 23 (100%) showed the presence of either *omp* or *htp* or both. Of these 17 (73.91%) showed amplification of both *omp* and *htp* target sequences. Four clinical isolates (17.39%) showed amplification of the *omp* gene, but not the *htp* gene, whilst two clinical isolates (8.69%) exhibited amplification of *htp* but not *omp*. This showed that most clinical *V. parahaemolyticus* isolates contained both genes. But both *omp* and *htp* need to be targeted in PCR tests to detect all known virulent strains tested. Out of 32 environmental isolates none contained both *omp* and *htp*, Only 3 out of 32 (9.37%) environmental isolates showed amplification for either *omp* (1 = 3.12%) or *htp* (2 = 6.25%) genes. No amplification of *omp* or *htp* sequences was seen in other *Vibrio* species or non-*Vibrio* species tested.

As described in Chapter 5, *V. parahaemolyticus* isolates were tested for cytotoxicity to the human Caco-2 and CHO-K1 cell lines and isolates that were positive for *omp* were strongly associated with cytotoxicity. It should be noted that isolates carrying *htp* were also associated with cytotoxicity (Table 6.3).

A blind test was performed on a randomly chosen collection of isolates including known clinical, environmental (avirulent) and other species relabelled by Dr. Martyn Gilpin. The blind test successfully identified isolates tested with regards to whether they were *V. parahaemolyticus* or not, and whether they were likely to be virulent or avirulent (Figure 6.17).
Figure 6.1 RAPD-PCR fragment patterns for genomic DNAs of clinical and environmental *V. parahaemolyticus* strains with primer pair P1 and P5.

Lanes 1, negative control; 2, *E. coli* BL21 (control DNA); 3, C2; 4, C4; 5, C5; 6, C7; 7, C8; 8, E16; 9, E22; Lane M, molecular size marker (100 bp DNA Ladder). Arrowhead indicates approximately 600-bp fragments commonly found in the virulent strains.
Figure 6.2 Agarose gel electrophoresis: (a) Recombinant *E. coli* plasmid after extraction; Lanes 1-4 plasmid extracted from blue colonies (non-recombinant); Lanes 5-8 plasmid extracted from white colonies (clones); (b) Restriction enzyme analysis to verify the presence of expected size inserts (band Y) within the plasmid of clones. M, 100bp; Lanes 1-4, non-recombinant; 5, C2, 6; C2; 7, C8; 8, C8.
**Chapter 6**

Vibrio parahaemolyticus RIMD 2210633 DNA, chromosome 1, complete sequence

Length = 3288558

Features in this part of subject sequence: putative outer membrane protein

Score = 1134 bits (614), Expect = 0.0

Identities = 617/617 (100%), Gaps = 0/617 (0%)

Strand = Plus/Plus

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**Figure 6.3** Nucleotide sequences of the gene *omp* identified in RAPD-PCR of clinical *V. parahaemolyticus* product.

152
Figure 6.4 Agarose gel electrophoresis of PCR amplified products using virulence-specific primers, VPOMP1 and VPOMP2.

Lanes M, 100-bp molecular mass marker. (a) Lane 1, negative control; Lanes 2-12 clinical *V. parahaemolyticus* as follow (2, C4; 3, C5; 4, C6; 5, C7; 6, C8; 7, C12; 8, C15; 9, C16; 10, C17; 11, C18; 12, C22); Lane 13, NCIMB Reference strain (R1); Lane 14, NCTC Reference strain (R2); Lanes 15-28 environmental *V. parahaemolyticus* as follow (15, E31; 16, E2; 17, E3; 18, E6; 19, E7; 20, E8; 21, E5; 22, E10; 23, E20; 24, E13; 25, E15; 26, E12; 27, E14; 28, E11). (b) Lanes 1-9 clinical as follow (1, C9; 2, C11; 3, C13; 4, C14; 5, C21; 6, C20; 7, C19; 8, C10; 9, C1); Lane10, negative control; Lanes (11-28) environmental *V. parahaemolyticus* as follow (11, E1; 12, E4; 13, E6; 14, E16; 15, E17; 16, E18; 17, E19; 18, E21; 19, E22; 20, E23; 21, E24; 22, E27; 23, E30; 24, E29; 25, E28; 26, E26; 27, E25; 28, E9). (c) Lanes 1- 2 clinical isolates as follow (1, C2; 2, C3); Lanes 3-24 Vibrio species as follow (3, VC; 4, VM; 5-10, VA1-VA6; 11-16, VA1-VA6; 17-21, VH1-VH5; 22-23, VN1-VN2; 24, VS; 25, negative control; Lanes, 26-30 non-Vibrio.
Figure 6.5 Alignment of the OMP sequence in isolates of *V. parahaemolyticus* RIMD 2210633 is a sequenced clinical strain under accession number BA000031.2.

Sequences E9 and C15 are from environmental and clinical isolates respectively and R1 is NCTC reference strain. Consensus regions are shown in red and denoted by asterisk; black and blue show deletions or substitutions.
Figure 6.6 Dot blot hybridisation of *V. parahaemolyticus* isolates and other species with Probe targeted OMP.
Table 6.2 *Vibrio parahaemolyticus* isolates tested shown results of PCR and dot blot

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+, Positive; -, negative; ( +/-), very weak
Figure 6.7 Agarose gel electrophoresis of RAPD-PCR: (a and b) RAPD single primer (OPK) screening of clinical isolates (C8 and C15) and environmental isolates (E2 and E22), Lanes M, 1Kb and 100bp Ladders.
Figure 6.8 Agarose gel electrophoresis of RAPD-PCR: (a-f) RAPD double primer screening of clinical isolates (C15 and C8) and environmental isolates (E2 and E22), Lanes M, 1Kb and 100bp Ladders.
Figure 6.8 continued
Figure 6.8 continued
Figure 6.9 RAPD-PCR fragment patterns for genomic DNAs of clinical and environmental *V. parahaemolyticus* isolate with primer pair OPK13 and OPK15.

(a) Lane 1 to 16 correspond to the following isolates: 1; NCIMB reference strain R1, 2; C16, 3; C18, 4; C19, 5; C22, 6; E26, 7; E5, 8; E27, 9; E29, 10; E30, 11; E31, 12; E1, 13; E6, 14; E3, 15; E12, 16; E13, 17, no primer control, 18, no template control; 19, *E. coli* BL21; 20, *E. coli* Cla. (b) Lanes 1 to 20 correspond to the following isolates: R2; 2, C2; 3, C4; 4, C5; 5, C7; 6, C10; 7, C14; 8, C11; 9, C3; 10, C12; 11, C1; 12, C9; 13, C13; 14, C21; 15, C17; 16, C6; 17, C20; 18, control without template. (c) Lanes 1 to 20 correspond to the following isolates: 1, R1; 2, E10; 3, E14; 4, E16; 5, E19; 6, E11; 7, E23; 8, E9; 9, E28; 10, E20; 11, E15; 12, E4; 13, E24; 14, E17; 15, E8; 16, E25; 17, E18, 18, E7; 19, E21; 20, control without template; Lanes M, 100 bp ladders with the sizes of selected markers indicated by the arrows.
Figure 6.9 continued
Figure 6.10 Reproducibility of RAPD fingerprinting profiles in 17 isolates of *V. parahaemolyticus*. Reference arrow on the left shows the position of an approximately 310 bp amplicon (band Z).
Figure 6.10 continued
Figure 6.10 continued
Figure 6.11 Agarose gel electrophoresis: (a) Recombinant *E. coli* plasmid after extraction; M, 1Kbp; Lanes 1-4 plasmid extracted from white colonies (clones); Lanes 5-6 plasmid extracted from blue colonies (non-recombinant); (b) Restriction enzyme analysis to verify the presence of expected size inserts (band Z) within the plasmid of clones. M, 100bp; Lane 1, C2; 2, C2; 3, C5; 4, C5; 5-6, non-recombinant.
Chapter 6

Features in this part of subject sequence:

**hypothetical protein**

Score = 568 bits (307/307 100%), Expect = 0.0
Identities = 307/307 (100%), Gaps = 0/307 (0%)
Strand=Plus/Plus

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Sbjct 1812456
TCTAATGACATCAGCAGCAACACGAACCCATTTCCAGGATTGGAGCAAGTAGAA

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Sbjct 1812696
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Figure 6.12 Nucleotide sequence of clinical *V. parahaemolyticus* isolate that produced band Z.

/translation="MSNDIQSARNSQP1HGLEQVEQRELAPQFTQGRKVTLLSSEN
KQRMSKRELSECLNQASLESCNQLVDFKEKPGTQAEKQAEKLDKVIARER". Predicting of the molecular weight of the protein sequence is 11.69 kDa.
Figure 6.13 Agarose gel electrophoresis of PCR amplified products using virulent specific primers, VPHTP1 and VPHTP2.

Lanes M, 100-bp molecular mass marker; (a) Lane 1, negative control; Lanes 2-12 clinical *V. parahaemolyticus* as follow (2, C3; 3, C4; 4, C5; 5, C1; 6, C9; 7, C2; 8, C6; 9, C11; 10, C7; 11, C10; 12, C8); Lanes 13-28 environmental *V. parahaemolyticus* as follow (13, R2; 14, E4; 15, E1; 16, E2; 17, E3; 18, E6; 19, E7; 20, E8; 21, E5; 22, E10; 23, E9; 24, E13; 25, E15; 26, E12; 27, E14; 28, E11). (b) Lane 1, negative control; Lanes 2-12 clinical as follow (2, C12; 3, C15; 4, C16; 5, C18; 6, C20; 7, C21; 8, C17; 9, C19; 10, C14; 11, C13; 12, C22); Lanes (13-27) environmental *V. parahaemolyticus* as follow (13, E18; 14, E19; 15, E23; 16, E24; 17, E29; 18, E30; 19, E16; 20, E17; 21, E25; 22, E26; 23, E31; 24, E28; 25, E27; 26, E22; 27, E21); Lane 28, *V. cholerae*. (c) Lane 1, negative control; 2, NCIMB Reference strain (R1); Lane 3, environmental isolate (E20); Lanes 4-28 *Vibrio* species and non-*Vibrio* as follow: (4, VM; 5-9, VA1-VA5; 10-11, VN1-VN2; 12, VS; 13-18, VA1-VA6; 19-23, VH1-VH5; 24-25, *Aeromonas*; 26, *E. coli*; 27, *K. pneumoniae*; 28, *Pro. vulgaris*.)
Figure 6.14 Alignment of the HTP sequence in isolates of *V. parahaemolyticus*. RIMD 2210633 is a sequenced clinical strain under accession number BA000031.2.

Sequences C16 and C8 are from clinical isolate and R1 is an NCTC reference strain. Consensus regions are shown in red and denoted by asterisk; black and blue show deletions or substitutions.
Figure 6.15 Hybridisation signals of *V. parahaemolyticus* isolates and other species with probe targeted HTP.
Figure 6.16 Agarose gel electrophoresis of multiplex PCR amplified products using three primer pairs.

Lanes M, 100-bp molecular mass marker. (a) Lane 1, control negative; 2-10 clinical isolates (2, R1; 3, C9; 4, C1; 5, C10; 6, C21; 7, C22; 8, C7; 9, C18; 10, C2); 11-20 environmental isolates (11, E9; 12, E3; 13, E1; 14, E2; 15, E10; 16, E30; 17, E31; 18, E4; 19, E5; 20, E8). (b) Lane 1, control negative; Lanes, 2-12 clinical isolates (2, C20; 3, C19; 4, C11; 5, C12; 6, C13; 7, C14; 8, C17; 9, C16; 10, C3; 11, C4; 12, C5); Lanes, 13-28 environmental isolates (13, E11; 14, E12; 15, E16; 16, E21; 17, E22; 18, E23; 19, E24; 20, E25; 21, E26; 22, E27; 23, E28; 24, E29; 25, R2; 26, E13; 27, E14; 28, E15). (c) Lane 1, control negative; Lanes 2-3 clinical isolates (2, C8, 3, C6); Lanes 4-9 environmental isolates (4, E20; 5, E19; 6, E18; 7, E17; 8, E6; 9, E7) lanes 10-20, other Vibrio species (10, VC; 11, VM; 12-17, VV1-VV6; 18-19, VN1-VN2; 20, VS. (d) Lane 1, control negative without template DNA; 2, clinical isolate (C15); 3-13 other Vibrio species (lanes, 3-8, VA1-VA6; 9-13, VH1-VH5); 14-17 Aeromonas; 18-24 non Vibrio (lanes 18-19, Pf1-2; 20, St; 21, Pv; 22, Kp; 23-24, Ec1-Ec2); 25, control negative without primers.
Table 6.3 List of *Vibrio parahaemolyticus* isolates tested in this study and results of multiplex PCR

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<td>4.0 L</td>
<td></td>
</tr>
<tr>
<td>E15</td>
<td>Environmental, UK</td>
<td>+</td>
<td>20.0 L</td>
<td>9.8 L</td>
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<tr>
<td>E16</td>
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<td>+</td>
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<tr>
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</tr>
<tr>
<td>E20</td>
<td>Environmental, UK</td>
<td>+</td>
<td>28.0 M</td>
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<tr>
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<td>+</td>
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<tr>
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<tr>
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<td>+</td>
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<tr>
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<td>+</td>
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<tr>
<td>E27</td>
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<td>+</td>
<td>19.0 L</td>
<td>5.0 L</td>
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<tr>
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<td>+</td>
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<tr>
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<td>5.0 L</td>
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</table>

1. H, Highly virulent; M, virulent; L, low virulence or avirulent.
Figure 6.17 Blind test for M-PCR, isolates A-U were subjected to the M-PCR and their likely identification is recorded below.

Lanes L, 100-bp molecular mass marker; lane A, Environmental.; B, Clinical; C, Environmental.; D, Environmental; E, Clinical; F, Clinical; G, Clinical; H, Environmental; I, Environmental; J, other species; K, Clinical; M, Clinical; N, Clinical; O, Environmental.; P, Environmental; Q, Clinical; R, Clinical; S, Environmental; T, Clinical; U, other species.
6.4 Discussion

6.4.1 RAPD-PCR using P1 and P5 primers

Molecular typing methods can be used to identify markers of virulence or pathogenicity for detection purposes, but before their use it is necessary to have gleaned which isolates are likely to be virulent or avirulent. In the case of *V. parahaemolyticus*, this information is likely to be related to the source of the isolates (i.e. clinical or environmental). Currently this information is corroborated by performing tests for the presence of putative virulence factors such as *tdh* and *trh*. Molecular typing tests such as pulsed-field gel electrophoresis (PFGE), ribotyping, tDNA, enterobacterial repetitive intergenic consensus (ERIC) or RAPD-PCR can then be used to identify markers which differentiate virulent and avirulent forms of the species. These methods can also be used for the characterisation of pandemic clones, for example, Khan et al., (2002) have used ERIC-PCR to identify the *V. parahaemolyticus* O3:K6-specific DNA segment.

Previous work done by Miah (2009) used RAPD-PCR to identify a unique DNA fragment produced in the majority of clinical isolates of *V. parahaemolyticus*. In the current study, this fragment has been utilised to create a PCR-based method for differentiating many clinical isolates from environmental isolates of *V. parahaemolyticus*.

RAPD-PCR with random primers P1 and P5 of most *V. parahaemolyticus* clinical isolates DNA showed a unique band Y at approximately 600 bp, which was purified from agarose gels, cloned and sequenced and used to design a specific primer set in this study. Using data from the sequence information for band Y, primer pair VPOMP1 and VPOMP2 was designed to amplify a 200 bp section of a gene encoding a yet uncharacterised outer membrane protein. This PCR band
was produced by 21 out of 23 (91%) of clinical (virulent) isolates tested and only 1 out of 32 environmental (mainly avirulent) isolates tested, the latter rate being in-line with previous studies to determine the likely capacity to cause disease amongst environmental strains (Cook et al., 2002, Hervio-Heath et al., 2002, Islam et al., 2004). While not 100% for all clinical isolates, this new PCR detected known virulent isolates better than any of the current individual PCR tests for \textit{tdh} and \textit{trh} positive strains. While the \textit{tdh} and \textit{trh} genes have been considered as a virulence marker genes of \textit{V. parahaemolyticus} for some time (Tada et al., 1992). Baffone et al. (2005) have found that a limited number of environmental strains of \textit{V. parahaemolyticus} are mostly responsible for \textit{V. parahaemolyticus} food-borne diseases.

The current study shows that of clinically recognised isolates of \textit{V. parahaemolyticus} tested 91% produced the 200 bp PCR-OMP product, suggesting that this new PCR test would be of value to detect strains potentially pathogenic to humans. These results support the notion that \textit{tdh} and \textit{trh} genes are not always present in pathogenic forms of \textit{V. parahaemolyticus} (Nishibuchi and Kaper, 1990).

Unfortunately, detailed information on the sources of the clinical isolates or indeed the immune status of the patients suffering from infection with \textit{V. parahaemolyticus} used in this study was not available. However, each was isolated from human cases of gastroenteritis. A possible explanation for the existence of clinical isolates that lack the 200bp PCR product (or any other putative virulence factor) is that some patients may have been immunocompromised and therefore, more susceptible to less virulent strains. Conversely, it should also note that a positive detection of a gene sequence by PCR does not necessarily mean that a gene product will be produced phenotypically. Likewise, a positive hybridisation and a
negative PCR could indicate differences in sequence at the primer binding sites. Moreover, it should be recognised that virulence in any bacterium will rarely be defined by a single gene.

Outer membrane proteins of bacteria play an important role during infection and induction of host immune response (Qian et al., 2008). Furthermore, the OMP NhhA of *Neisseria meningitides* is important for colonisation and meningococcal disease, the importance of this protein has been studied *in vitro* where this NhhA mediates attachment of bacteria to cells, as well as *in vivo*, the impact of NhhA on pathogenesis of bacteria has been demonstrated in a murine model of meningococcal disease by showing lethality using CD6 transgenic mice (Sjölinder et al., 2008). Krachler et al., (2011) have reported that a multivalent adhesion molecule has been found as an OMP in *V. parahaemolyticus* and it has a role in attachment to Caco-2 and HeLa cells, suggesting that contributes to pathogenesis.

While the current study developed a PCR which detects nearly all virulent *V. parahaemolyticus* isolates, it is realised that the number of isolates used is relatively small and thus represents a preliminary, but informative, study. The 200 bp PCR-OMP offers the possibility of screening environments or seafood and other foodstuffs for the presence of potentially pathogenic forms of *V. parahaemolyticus*. Furthermore, the nature and role of this OMP in this bacterium and its contribution to virulence needs to be studied more.

Sequencing of PCR products and sequence analysis for homology was performed to verify the identity of PCR products and confirm the results. Sequence analysis of three isolates showed that single nucleotide polymorphisms (SNPs) did not alter the translated amino acid.
In this study, it was necessary to confirm a PCR-OMP product (200bp) by hybridisation analysis, very few isolates were positive for hybridisation and negative for PCR that may be caused by nucleotide mismatches or a lack of primer target sites or other reasons (Miah, 2009).

**6.4.2 RAPD-PCR using OPK primers**

In recent years, a number of molecular methods have been used for detection and identification of *V. parahaemolyticus*. In this study, RAPD-PCR was used with random primers OPK13 and OPK15 to identify a target DNA sequence which differentiated most clinical and environmental tested. To the author’s knowledge, there did not appear any publish studies that used this random primer pair. Most clinical isolates produced a unique band at approximately 310 bp (band Z), which was purified from agarose gels, cloned and sequenced. This sequence codes for a hypothetical protein whose function is currently not known. A specific primer set VPHTP1 and VPHTP2 was designed to specifically amplify a 300 bp section of the gene *htp* encoding a hypothetical protein. This 300 bp PCR product was produced by 19 out of 23 (82.6%) of clinical (virulent) isolates tested and only 2 out of 32 environmental (mainly avirulent) isolates. No band was seen with any of the other species of *Vibrio* and non-*Vibrio* strains tested.

Okura et al., (2004) have similarly used arbitrarily primed- PCR with random primer P2 (5’-d[GTTCGCTCC]-3’) to investigate a unique band (approximately 930 bp) in pandemic strains of *V. parahaemolyticus* corresponding to a hypothetical protein, which was found to be 80% homologous to the Mn2+ and Fe2+ transporter of the NRAMP family of *V. vulnificus*. Okura et al., (2004) then designed primers to detect a specific amplicon at 235 bp, which was a useful
diagnostic tool to distinguish the pandemic group from other *V. parahaemolyticus* strains.

The results of the current study suggest that RAPD shows promising ability to differentiate between clinical and environmental (predominantly avirulent) isolates of *V. parahaemolyticus*. It should be noted that some environmental isolates will have the capacity to cause disease. It may be that different primer sets might be able to distinguish virulent from avirulent isolates completely and be developed into a quick PCR based test.

### 6.4.3 Multiplex PCR

This study developed a multiplex PCR to identify all *V. parahaemolyticus* isolates tested by producing species specific fragment *toxR* gene (367 bp) and reveal potentially virulent forms by detecting either *omp* (200 bp) or *htp* (300 bp) sequences or both. Thus the multiplex PCR method was simultaneously used to identify *V. parahaemolyticus* and targeted both *omp* and *htp* genes in a single PCR reaction. This multiplex PCR detected 3 (9.37%) in environment isolates.

The presence of both *omp* and *htp* correlated with high cytotoxicity. Isolates which did not carry either *omp* or *htp*, i.e. the majority of the environmental isolates, showed low cytotoxicity, with one exception, the NCTC reference strain R2. This is environmentally sourced and did not give a positive PCR for either *omp* or *htp*, but showed a level of cytotoxicity on a par with many of the clinical (virulent) isolates tested here. It is likely that either *omp* or *htp*, or both, act merely as markers for cytotoxicity, i.e. are not directly responsible for cytotoxicity. Perhaps they are located on a genetic region or element which when acquired also carries a gene or genes for cytotoxic products. Maybe in R2 the marker genes have become detached and lost from any cytotoxic genes, but the latter remain and are active.
Alternatively, R2 may contain a hitherto new or separate cytotoxic activity, unrelated to *omp* or *htp*.

Previously, between 6% (Shirai et al., 1990, Cook et al., 2002) and 9% (Cabrera-Garcia et al., 2004) of environmental *V. parahaemolyticus* isolates have been designated as potentially pathogenic, so the findings of the current study are in line with others.

This PCR shows great promise to be used as a suitable tool to identify *V. parahaemolyticus* and detect potentially virulent forms. Moreover, it could be used in epidemiological studies and investigations into the abundance of potentially virulent forms of *V. parahaemolyticus* in various environmental habitats.
CHAPTER 7

GENERAL DISCUSSION AND RECOMMENDED FUTURE WORK
Chapter 7: General Discussion and Recommended Future Work

7.1 General discussion

*Vibrio parahaemolyticus* is a human gut pathogen associated with ingestion of seafood. Only certain strains of the species, carrying virulence factors rarely found in environmental strains, cause disease in humans that associated with consumption of seafood causing acute diarrhoea and vomiting. However, it is likely that most *V. parahaemolyticus* isolates from the marine environment are harmless. Virulent strains of *V. parahaemolyticus* have become a major cause of food borne disease. Therefore, the extensive studies of this bacterium are interesting and necessary because identification and detection of the virulent forms of human pathogenic *Vibrio* species such as *V. parahaemolyticus* in the seafood samples is important in the food industry.

It is important to know whether food samples contain pathogenic or non-pathogenic bacteria. For example, the presence of pathogenic *E. coli* O157:H7 in food is a danger and can cause disease (Karmali, 2009). If the batches of food contain only non-pathogenic strains that means these strains are harmless and should not result in discard of that food batch. Likewise, virulent and avirulent *V. parahaemolyticus* exist, therefore, if *V. parahaemolyticus* is present in batches of seafood, it is necessary to know whether it is virulent or avirulent, instead of discarding the batches straight away. It appears that there is no single genetic determinant necessary and sufficient for virulence of this bacterium to occur (Yeung and Boor, 2004), and many studies have reported that there are likely to be many factors involved in *V. parahaemolyticus* pathogenicity.

This thesis focused on the development of methods for differentiating between virulent (mainly clinical) and avirulent (mainly environmental) isolates of *V. parahaemolyticus*.
parahaemolyticus. Virulence was gauged by the capacity of the isolates to produce cytotoxic extracellular products and lyse mammalian cell lines. The LDH assay detected cell lysis and differentiated between virulent (clinical) and mainly avirulent (environmental) isolates of this bacterium and could therefore be used to screen for the presence of potentially virulent forms in the environment. The phenotypes and genotypes of isolates tested could be associated with the enhanced cytotoxic effects of *V. parahaemolyticus* isolates tested.

In many European countries, pathogenic *Vibrio* species are present in estuarine and coastal water and in shellfish (Barbieri et al., 1999, Baffone et al., 2001, Hervio-Heath et al., 2002, Cavallo and Stabili, 2002). In recent years, the incidence of *V. parahaemolyticus* infections and the risk by this pathogen have increased dramatically in Europe, for example in Spain and France (Martínez-Urtaza et al., 2005, Quilici et al., 2005). Pathogenic *V. parahaemolyticus* has been detected in French bivalve molluscs in France by Rosec et al., (2009). *V. parahaemolyticus* has also been detected in blue mussels (*Mytilus edulis*) that harvested from the German Wadden Sea, suggested that potentially pathogenic *V. parahaemolyticus* has considered as a public health and potential human health risk (Lhafi and Kühne, 2007). Another study in Norway has reported that potential pathogenic *V. parahaemolyticus* has been detected in *M. edulis* (Bauer et al., 2006). Furthermore, Serracca et al., (2011) have detected pathogenic strains of *V. parahaemolyticus* in fishes collected from estuarine waters in Italy and reported that the potential human health risk related with consumption of raw or undercooked fishes.

Response of *V. parahaemolyticus* to environmental conditions such as climate change provides a valuable insight into understanding increasing levels of this bacterium in Europe, high temperatures and low salinity have been led to increase
incidence of potential virulent *V. parahaemolyticus* in European countries such as in Spain (Martinez-Urtaza et al., 2008), France (Julie et al., 2010) and Brazil (Sobrinho et al., 2010). Furthermore, Collin and Rehnstam-Holm, (2011) have reported that potential human pathogenic *Vibrio* species including *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* in the Sound between Sweden and Denmark are common at water temperature above 17 °C.

Investigating the presence of virulence factor determinants by PCR shows that the presence of the *tdh* gene or *trh* gene, (or both) are considered as diagnostic markers for pathogenic isolates of *V. parahaemolyticus* (Nordstrom et al., 2007). However, it has been noted that these virulence genes are absent in some pathogenic strains. Thus, some *V. parahaemolyticus* isolates lack TDH and TRH, but remain pathogenic (Nishibuchi et al., 1992, Xu et al., 1994, Park et al., 2000).

Recently, *V. parahaemolyticus* strains lacking *tdh* and/or *trh* caused outbreaks in Southern Chile (García et al., 2009) therefore, there is a need to investigate other possible genes responsible for their virulence. Conventional phenotypic and molecular techniques are widely used for identification and detection of total and virulent strains of this organism. PCR is extensively used to help conventional bacteriological tests, and it gives benefit for identification of *Vibrio* species and detection of pathogenic strains. So while, TDH has been recognised as a marker for discriminating pathogenic from non-pathogenic strains (Honda and Iida, 1993) it does not do so completely. Moreover, Bilung et al., (2005b) isolated 62 *V. parahaemolyticus* from 100 samples of cockles in Malaysia and detected by PCR the *tdh* gene in only 2 isolates and *trh* gene in only 11 isolates. Vongxay et al., (2008) have reported that the *trh* gene has been detected in 3 out of 25 clinical and 7 out of 191 environmental isolates of *V. parahaemolyticus*; and they
suggested that there are factors other than TDH for virulence. Although some isolates of *V. parahaemolyticus* lack the *tdh* gene they still appear to be pathogenic; however, the overall mechanism associated with pathogenicity of this bacterium is not understood and remains unclear (Makino et al., 2003, Boyd et al., 2008). Consideration of the *tdh* gene as a marker to predict the virulent forms of *V. parahaemolyticus* is better than the *trh* gene has been reported (Kaper et al., 1984). Studies have reported that other *Vibrio* species also harbour *trh*-like genes highly homologous with *trh* gene from *V. parahaemolyticus* for example, it has been detected in *V. alginolyticus* (Gonza´Lez-Escalona et al., 2006, Rosec et al., 2012) and *Aeromonas veronii* (Raghunath et al., 2010). Furthermore, Masini et al., (2007) have detected *trh* gene in *V. alginolyticus* and *V. harveyi* isolated from bathing water Conero Riviera. Similar observation was reported by Nishibuchi and Kaper, (1995) for *tdh* gene and *V. hollisae, V. mimicus* and *V. cholerae non-O1*.

In the current study, the putative virulence factors of *V. parahaemolyticus* isolates have been investigated and surveyed as possible virulence factors to find patterns that might distinguish clinical (virulent) and environmental (mainly avirulent) isolates and to identify better markers for distinguishing these groups (Chapter 4), the results showed that 14 isolates from clinical sources and one environmental isolate could produce haemolysis, urease, protease and phospholipase, however, lipase activity did not appear to contribute to the pathogenicity of the isolates tested. High cytotoxicity remained evident in virulent strains and was considered as quantitative evidence for the virulence of these isolates. It could be seen that most of phospholipase positive isolates produced haemolytic activity, and most isolates that were positive for these two activities showed high cytotoxicity but there are isolates which did not produce these two enzymes but showed high cytotoxicity, for example C17 isolate showed cytotoxicity of 65% and 52% on
Caco-2 and CHO-K1, respectively (Chapter 5), suggesting that haemolytic activity and phospholipase activity may play a role in most, but not all, of *V. parahaemolyticus* pathogenicity.

However, in the current study, there were some protease positive environmental isolates which showed less than 20% cytotoxicity on Caco-2 and CHO-K1, suggesting that the protease enzyme in these isolates might not be involved in cytotoxicity or in other isolates a different protease plays a role in pathogenicity. Likewise all clinical isolates and 15 environmental isolates were positive for urease, but some of these urease positive environmental isolates showed low cytotoxicity. It would appear that urease activity is essential for pathogenicity but not cytotoxicity.

Plasmids and bacteriophages (Chapter 4) were also studied to investigate if their presence in *V. parahaemolyticus* isolates tested was linked to the carriage of virulence factors. The presence of plasmids was found not to be correlated with virulence factors and pathogenicity and therefore of no use to differentiate between clinical and environmental isolates of *V. parahaemolyticus*. Twedt et al., (1981) isolated plasmids in 2 of 14 KP+ and 1 of 17 KP− and suggested that the presence of plasmid did not correlate with the pathogenicity of *V. parahaemolyticus* isolates they tested. Furthermore, Isaki et al., (1983) have reported that there was no correlation found between the presence of plasmid and KP reaction.

When SDS-PAGE was used to analyse whole cell protein bands, protein profiles could not be used to differentiate clinical (virulent) and environmental (mainly avirulent) isolates of *V. parahaemolyticus*. The whole cell protein patterns of
clinical *V. parahaemolyticus* are generally identical to environmental isolates. There are some drawbacks of standard SDS-PAGE method describing as follow:

1- It is hard to detect two proteins with similar size apart from each other.

2- A complex protein mixture with some abundant protein, it is hard to detect less abundant proteins if they are about the same size of the abundant ones.

3- The SDS treatment denatures the protein and gives the protein negative charge; it does not detect a protein in its natural state.

Studying genomes of bacteria have contributed to understand the mechanisms that involved in their virulence and pathogenicity by generating and comparing whole genome sequencing, this started in mid-1990 with the automation of Sanger sequencing and the completion of *Haemophilus influenza* (Fleischmann et al., 1995) and *Mycoplasma genitalium* (Fraser et al., 1995). High throughput sequencing has increased the amount of completed genomes of bacteria and the speed of DNA sequencing. This technology has been used to investigate the origins and potential pathogenic of *E. coli* O104:H4 that caused outbreak in Germany in 2011 (Rasko et al., 2011, Rohde et al., 2011), and *V. cholerae* that caused cholera outbreak in Haiti (Chin et al., 2011). Furthermore, Gonzalez-Escalona et al., (2011) have used this technology to sequence genome of clinical *V. parahaemolyticus* strain 10329.

There are several molecular typing systems for investigating the differences between the bacterial isolates; however, the main criteria to choose the typing methods are their reproducibility, typeability, constancy, cost and simplicity of performance. PFGE is a gold standard technique that has been used for molecular typing of *V. parahaemolyticus* (Martinez-Urtaza et al., 2004), but it is expensive
and time-consuming. RAPD-PCR is quick, cheap, relatively simple to achieve and was used in this study to initially differentiate *V. parahaemolyticus* clinical (virulent) isolates from the environment (mainly avirulent) isolates (Chapter 6). It has been found that an outer membrane protein and a hypothetical protein may play a role in the pathogenicity of virulent strains of *V. parahaemolyticus* or are markers for pathogenic strains.

The outer membrane protein (OMP) of enteric bacteria is one of the numerous factors that many allow attachment of enteric pathogens to the intestinal epithelium of the host (Nikaidol and Vaara, 1985). Aeckersberg et al., (2001) have reported that the relationship between OMPs of *Vibrio* and pathogenicity is an important issue, but it remains unresolved yet. OMPs of *V. fischeri* act as adhesions and play a role in symbiotic colonisation (Aeckersberg et al., 2001). The OMP of *V. harveyi* contributes to bacterial adherence (Ningqiu et al., 2008). Provenzano and Klose, (2000) have reported that OMPs are important for *V. cholerae* pathogenesis providing bile salt resistance and survival of the bacteria in the intestine. Sperandio et al., (1995) and Chakrabarti et al., (1996) have suggested that OmpU acts a potential adherence factor in *V. cholerae*. On the contrary, Nakasone and Iwanaga (1998) have suggested that OmpU does not contribute to the adhesion of this pathogen to the epithelia cells. OmpU is also reported to play an important role in the pathogenicity of *V. splendidus* in oysters (Marylise et al., 2010). An OMP has also been implicated in the disease processes of *V. tubiashii* (Beaubrun et al., 2008). Moreover, Broberg et al., (2011) have reported that an OMP was essential for preliminary contact of *V. parahaemolyticus* with various cell lines.
Chapter 7

The OMP-PCR and the HTP-PCR separately did not detect all the known clinical virulent forms tested. Because of this limitation, in the current study, a M-PCR was developed to allow the simultaneous detection of toxR, omp and htp in a single tube, with the expected amplicon sizes of 367 bp for toxR, 300 bp for htp and 200 bp for omp using 3 pairs of primers. This M-PCR was able to identify V. parahaemolyticus and detect all the virulent forms tested so far. A number of workers have found that a M-PCR approach is a useful and rapid tool, for example, development of M-PCR detection of haemolysin genes (tlh, tdh and trh) was reliable and quicker than bacteriological methods for monitoring and occurrence of virulent strains of V. parahaemolyticus in seafood in Turkey (Terzi et al., 2009). Another M-PCR developed by Kaufman et al., (2002) targeting three genes (tlh, tdh and trh) along with urease production could be used as a useful tool for monitoring V. parahaemolyticus in shellfish and detect potential pathogenic strains.

The cytotoxic effect of ECPs of V. parahaemolyticus isolates has been tested in the present study. In general, the clinical isolates exhibited greater LDH release from Caco-2 and CHO-K1 than the majority of environmental isolates. However, the strains of V. parahaemolyticus that were positive for the 200 bp (omp) or/ and 300 bp (htp) PCR product caused significantly \( p < 0.05 \) more (the mean of the percentage of cytotoxicity values 67.4%) than for those that lacked these two fragments (mean 13.9%). Another cell line, CHO-K1 was also used for cytotoxicity test and found that the strains of V. parahaemolyticus that were positive for the OMP-PCR or/ and HTP-PCR caused also significantly \( p < 0.05 \) more (the mean of the percentage of cytotoxicity values 57.4%) than for those that lacked these two fragments (mean 8.9%). Thus by using Caco-2 and CHO-K1 cell lines, it was shown that the mean of the percentage of cytotoxicity of V. parahaemolyticus ECPs was significantly greater for isolates that produced the 200 bp PCR-OMP...
or/and 300 bp PCR-HTP products than for those that lacked these fragments, however, the environmental isolate (E20) carried the htp gene, but lacked the omp gene but exhibited a certain degree of cytotoxicity 28% on Caco-2 and 29.5% on CHO-K1 cells. Importantly, these results found quantitative evidence for enhanced virulence-associated characteristics of OMP and HTP group isolates compared to those of other *V. parahaemolyticus* strains, since the quantity of LDH released is relative to the degree of the host cell membrane damage (Nachlas et al., 1960). Based on cytotoxicity to Caco-2 cell line, Yeung et al. (2007) also differentiated virulent and avirulent strains and the cytotoxicity of clinical isolates was significantly different (*p* ≤ 0.05) from food isolates. Raimondi et al. (2000) reported that the high TDH concentration had been demonstrated to induce cytotoxicity in Caco-2 cells, but in the present study there is a high cytotoxic effect of ECPs in isolates that produced the 200 bp or/and 300 bp fragments even though in some instances, one or more of the *tdh* and *trh* genes may have been absent.

The present work suggested that the cytotoxicity test based on LDH release from two types of cell lines was useful to determine the virulence of *V. parahaemolyticus* isolates; however, the presence of a single virulence gene alone or production of one of the putative virulence factors alone cannot be used as a mean for determining the potential virulence of strains, suggesting that multiple virulence factors contribute to the pathogenicity of this bacterium.

Avirulent strains of *Vibrio* species are considered to provide a main reservoir of known virulence genes. The mobility and successful transfer of virulence genes may lead to new virulent strains by transformation of avirulent forms to virulent strains. For example, the *tdh* gene can be transferred horizontally between *V. hollisae* and *Vibrio* other species including, *V. cholerae*, *V. mimicus* and *V.
*parahaemolyticus* (Nishibuchi et al., 1996). The marine habitat harbours many virulence genes scattered among vibrios in the environment (Xie et al., 2005).

The presence of a number of virulence related genes have been reported in both clinical and environmental isolates of *V. parahaemolyticus*. However, the relationship between *V. parahaemolyticus* isolated from the marine environment and from clinical sources is unclear (Pan et al., 2007). The pathogenicity of *V. parahaemolyticus* may perhaps involve the synergistic interactions between several factors that have been studied in the current work. Furthermore, some clinical *V. parahaemolyticus* isolates from patients who are immunocompromised or have underlying liver disease or those with diabetes mellitus may lack one or more known putative virulence factors, but still cause disease in these individuals.

The current M-PCR suggested that 9.37% of environmental isolates tested are likely to be potentially virulent. This result is supported by many studies that report that amongst environmental isolates potential pathogen isolates, exist at a low rate, for example 5% (Hervio-Heath et al., 2002), 6% (Caburlotto et al., 2009) and 10.5% (Rojas et al., 2011).

The M-PCR assay was successfully used to identify *V. parahaemolyticus* and detect potentially virulent forms targeting three genes simultaneously in a single reaction, and it is a rapid and cheap method for the seafood industry and would reduce the infection caused by *V. parahaemolyticus* by ensuring seafood safety, and reduce waste by only removing batches of food that had virulent *V. parahaemolyticus* present.
7.2 Recommended future work

The studies described in this work have not included isolates from the entire world, thus a larger number of clinical and environmental of *V. parahaemolyticus* isolates from more diverse geographical sources would be recommended for future work.

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 CTXø bacteriophages carrying the cholera toxin that is found in O1 and O139 serotypes of *V. cholerae* (Waldor and Mekalanos, 1996, Faruque et al., 1998).

Further analysis of the exact nature of extracellular proteins produced by *V. parahaemolyticus* may well unravel the virulence exhibited by this organism. Define the functional role of ECPs. The ECPs need to be investigated further by purification and identification of proteins in ECPs, and to study their biological activities. The determination of whether ECPs production plays a crucial role in the disease needs to be studied in vivo as well as in vitro; using fish infection experiments instead of using cell lines.

Further work is required to investigate whether *omp* or *htp* are virulence factors or merely markers of virulence. Determining the contributions of OMP and HTP and their possible role in pathogenicity needs to be studied more. The role of *omp* and *htp* genes in virulence may be investigated by creating mutants within the *omp* or/and *htp* genes of the virulent isolates and test them for cytotoxicity.

Although the reliability of this M-PCR needs further evaluation with more clinical and environmental strains, this type of PCR may well prove to be the most
accurate way to identify *V. parahaemolyticus* and differentiate potentially so far devised virulent forms.

Development of NGS technologies to assess the distinct genomic differences between pathogenic and non-pathogenic strains of *V. parahaemolyticus* is needed in future work.
APPENDIX I

MEDIA, REAGENTS, SOLUTIONS AND SUPPLIERS
Appendix I: Media, Reagents, Solutions and Suppliers

I.1 Bacteriological media

The bacteriological media were sterilized by autoclaving under these conditions, 15 pounds of pressure per square inch (Psi) and 121 °C.

**Luria-Bertani medium (LB)**

Per litre of ddH$_2$O (pH 7.5):

- Tryptone 10 g
- Yeast extract 5 g
- Sodium Chloride 10 g

Contained the same components as stated in the LB media although LB agar with the addition of 1.5% (w/v) agar

**Marine salts broth (MSB)**

Marine Broth 2216 powder was purchased as pre-mixed media from Becton Dickinson, distributors for Difco Labs. A 37.4 g of medium was dissolved in 1L of ddH$_2$O, boiled for 1 min to completely dissolve the powder and autoclaved.

Approximate formula per litre (pH 7.6 at 25 °C)

- Peptone 5.00 g
- Yeast Extract 1.00 g
- Ferric Citrate 0.10 g
- Sodium Chloride 19.45 g
- Magnesium Chloride 5.90 g
- Magnesium Sulphate 3.24 g
- Calcium Chloride 1.80 g
- Potassium Chloride 0.55 g
- Sodium Bicarbonate 0.16 g
- Potassium Bromide 0.08 g
- Strontium Chloride 34.0 mg
- Boric Acid 22.0 mg
- Sodium Silicate 4.0 mg
- Sodium Fluoride 2.4 mg
- Ammonium Nitrate 1.6 mg
- Disodium Phosphate 8.0 mg
**Marine salts agar (MSA)**

Marine Broth 2216 powder (37.4 g) of media was dissolved in 1L of ddH$_2$O with the addition of 1.5% w/v agar.

**Tryptone soya broth (TSB)**

TSB powder was purchased as pre-mixed media from Oxoid; 30g of media was dissolved in 1 L of ddH$_2$O, the pH adjusted to 7.5 and then autoclaved. The formula of the resulting media is as follows:

- Tryptone (casein digest USP) 17.0 g
- Soya peptone 3.0 g
- Sodium chloride 5.0 g
- Dibasic potassium phosphate 2.5 g
- Dextrose 2.5 g

**Tryptone soya agar (TSA)**

TSA powder was purchased as pre-made media from Oxoid. 37g of media was dissolved in 1 L of ddH$_2$O, the pH adjusted to 7.5 and then autoclaved. TSA contained the same components as stated in the TSB media although TSA with the addition of 1.5% (w/v) agar.

**TCBS agar**

TCBS Cholera Medium (CM0333) powder was purchased as pre-made media from Oxoid. A 88 g of media was dissolved in 1 L of ddH$_2$O (pH 8.6 ± 0.2 at 25°C). The formula of the resulting media is as follows:

- Yeast extract 5.00 g
- Bacteriological peptone 10.00 g
- Sodium thiosulphate 10.00 g
- Sodium citrate 10.00 g
- Ox Bile 8.00 g
- Sucrose 20.00 g
- Sodium chloride 10.00 g
- Ferric citrate 1.00 g
- Bromothymol blue 0.04 g
- Thymol blue 0.04 g
- Agar 14.0 g
**HiCrome™ Vibrio agar**

HiCrome™ Vibrio Medium (92323) powder was purchased as pre-mixed from Sigma. A 67.5g of media was dissolved in 1 L of ddH$_2$O. The formula of the resulting media is as follows:

- Peptic digest of animal tissue: 10.0 g
- Sodium chloride: 25.0 g
- Sodium thiosulfate: 5.0 g
- Sodium citrate: 6.0 g
- Sodium cholate: 1.0 g
- Chromogenic mixture: 5.5 g
- Agar: 15.0 g

pH 8.5 ± 0.2 at 25°C

**Wagatsuma agar (WA)**

- Yeast extract: 3 g
- Peptone: 10 g
- NaCl: 70 g
- Mannitol: 10 g
- Crystal violet: 0.001 g
- Agar: 15 g

All above ingredient, except blood, was suspended in distilled water (950 ml) and boiled to dissolve agar. The pH was adjusted to 8.0 and steam sterilised (30 min). A volume of 50 ml of rabbit blood cells was added to the cooled (45 ºC) medium.

**Phospholipase assay medium**

Sudheesh and Xu, (2001)

- peptone: 10 g
- NaCl: 30 g
- Agar: 15 g
- Egg yolk: 2.5%

The medium was made up with distilled water to 1L. All above ingredient, except Egg yolk, were suspended in distilled water and boiled to dissolve agar and autoclave (30 min). A 2.5% of Egg yolk Emulsion was added to the cooled (45 ºC) medium.
Urea agar base medium
Based on Atlas, (1997)

NaCl  30 g
Na₂HPO₄ 1.2 g
Pepton  1 g
Glucose 1 g
KH₂PO₄ 0.8 g
Agar 15 g
Distilled water 950 ml
Urea solution 40%

All above ingredient, except urea solution, was suspended in distilled water 950 ml and boiled to dissolve agar, mixed thoroughly and autoclave (30 min). A volume of 50 ml of Urea solution was added to the cooled (45 °C) medium.

Protease assay medium
Depended on Wong et al., (1992)

Skim milk 15 g
NaCl 30 g
Agar 15 g

The medium was made up with distilled water to 1L

Lipase assay medium
Depended on Wong et al., (1992)

Peptone 10 g
NaCl 30 g
Agar 20 g
CaCl₂, H₂O 0.1 g
Tween 80 1 %

The medium was made up with distilled water to 1 L

SOC medium (100 ml), filter sterilize, pH 7.0

Tryptone 2 g
Yeast extract 0.5 g
NaCl 1M 1 ml
KCl 1M 0.25 ml
Glucose 2M 1 ml

Stock Mg²⁺ 2M containing (MgCl₂.6H₂O, 20.33 g and MgSO₄.7H₂O, 24.65 g).
I.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) solutions:

**Resolving gel buffer**
Trizma-base 0.75 M (pH 8.8)
SDS 0.2% w/v

**Stacking gel buffer**
Trizma-base 0.25 M (pH 6.8)
SDS 0.2% w/v

**SDS gel-loading buffer**
Trizma-HCl 0.5 M (pH 6.8)
SDS 2%
Bromophenol blue 0.1%
2-mercaptoethanol 5%
Glycerol 10%

**Electrophoresis buffer-1000 ml (pH 8.3)**
Trizma-base 25 mM
Glycine 250 mM
SDS 0.1%

**Resolving gel 12.5 %**
Acrylamide/bis-acrylamide 30%, 4.4 ml
Resolving gel buffer 5.2 ml
ddH$_2$O 1.74 ml
APS 10%, 60 µl
TEMED 30 µl

**Stacking gel 5%**
Acrylamide/bis-acrylamide 30%, 0.7 ml
Stacking gel buffer 2 ml
ddH$_2$O 1.3 ml
APS 10%, 50 µl
TEMED 15 µl

**Staining**
Commassi brilliant blue R-250 0.125%
Methanol 40% (v/v)
Acetic acid 7% (v/v)

**Destained**
Methanol 40% (v/v)
Acetic acid 7% (v/v)

1.3 Extraction of genomic DNA
Bacterial genomic DNA was extracted by using a genomic DNA extraction kit (Qiagen, Ltd., UK). Details of the contents of each solution are unknown.

1.4 Cytotoxic activity

**Lysis solution (10X)**
Triton®X-100 9% (v/v) in water

**Stop solution**
Acetic acid 1M

**Substrate mixture**
L-(+)-lactic acid 0.05M
$p$-iodonitrotetrazolium violet $7 \times 10^{-4}$ M
Nicotinamide Adenine Dinucleotide Sodium (NAD) $3 \times 10^{-2}$ M in 0.2 M Tris buffer (pH 8.2)

**Dulbecco's Modified Eagle Medium (D-MEM) (1X) without Phenol red**
D-glucose 4.5 g/l
L-glutamine 5 mM
HEPES buffer 25 mM

**Dulbecco's Modified Eagle Medium (D-MEM) (1X) with Phenol red**
D-glucose 4.5 g/l
L-glutamine 5 mM
Sodium Pyruvate 110 mg/l
Phenol Red Indicator

**Trypsin, 0.25% (1X) with EDTA 4Na, liquid**
Trypsin is widely used for dissociation of tissues and cell monolayers
Trypsin (1:250) 2.5 g/l
EDTA.4Na 0.38 g/l
Phenol Red
Hanks' Balanced Salt Solution without CaCl₂, MgCl₂

**Dulbecco’s Phosphate Buffered Saline (D-PBS) (1X), liquid**
Calcium Chloride (CaCl₂) (anhyd.) 0.901 mM
Magnesium Chloride (MgCl₂-6H₂O) 0.493 mM

**I.5 Plasmid extraction solutions**

**A- Bimboim and Doly, (1979)**

**B&D solution I**
Glucose 50 mM
Trizma HCl 25 mM
EDTA 10 mM
pH 8.0

**B&D solution II**
NaOH (1 ml) 0.2 M
SDS (1 ml) 1 M

**B&D solution III**
Potassium acetate (60 ml), 5 M (pH 4.8)
Glacial acetic acid (11.5 ml)
dd H₂O (28.5 ml)

**B- Pedraza and Díaz Ricci, (2002):-**

**Lysis buffer:**
This solution was prepared by using TBE buffer
Sucrose 10 g
RNase 10 µg
Lysozyme 1 µg

**I.6 General molecular biology reagents**

**Gel loading solutions**
(1) General gel loading (x6) solution (Sigma):
Bromophenol blue 0.25% (w/v)
Xylene cyanole FF 0.25% (w/v)
Sucrose 40% (w/v)

(2) Tracking buffer (x6) for RAPD (Amersham Biosciences)
Glycerol 30%
Bromophenol blue 0.125%
Tris-HCl 20 mM (pH 8.0)

**TE buffer, pH 8.0**
Tris-base 10 mM
EDTA 1 mM

**TBE buffer (5x)-1000 ml**
TBE was made up at 5X strength according to the following recipe and was then
diluted to 1 X:
Trizma base 54 g
Boric acid 27.5 g
EDTA 0.5 M (pH 8.0) 20 ml

I.7 PCR

10x PCR reaction buffer
10 x buffer consisted of (15 mM MgCl₂, 100 mM Tris-HCl, 500 mM KCl, pH 8.3)

**PCR Grade Nucleotide**
10 mM dNTP solution

**Phosphate buffered saline**
One tablet (Oxoid) dissolved in 100 ml ddH₂O. Sterilised by autoclaving as
mentioned in section 1.1.

I.8 Blue/White Screening

**X-Gal solution**
5-bromo-4-chloro-3-indolyl -β-D-galactoside (X-gal) made up as stock solution 50
mg/ml in N, N’ -dimethylformamide (DMF) and sterilized by filtration (Whatman,
0.45 µm) and covered with aluminium foil and stored at -20 °C.

**IPTG solution**
Isopropyl -β-thiogalctopyranoside (IPTG) made up as stock solution 100 mM in
molecular biology water. Filter sterilized and stored at 4 °C.
**LB plate with ampicillin/X-Gal/IPTG**

LB medium was prepared as described in section I.1. A volume of 20 µl of X-Gal, 100 µl of IPTG and ampicillin at final concentration 100 µg/ml were added to the cooled (45 °C) medium.

**I.9 Agarose Gel DNA Extraction Kit**

The kit contents are as follows:

**Silica matrix**

Vial (1) with a pretreated spherical silica matrix

**Agarose solubilisation buffer**

Vial (2) with 60 ml agarose solubilisation buffer contains sodium perchlorate.

**Nucleic acid binding buffer**

Vial (3) 100 ml binding buffer contains sodium perchlorate.

**Washing buffer**

Vial (4) with 20 ml washing buffer, add 80 ml absolute ethanol

**I.10 PCR product purification kit solutions**

PCR product was purified using the ‘High Pure PCR Product Purification Kit’ supplied by Roche; all buffers were kept at room temperature. Details of the content of each solution are as follows:

**Binding Buffer**

Guanidine-thiocyanate 3 M
Tris-HCl 10 mM
Ethanol 5% (v/v)

pH 6.6

**Washing Buffer**

(Final concentrations after adding of 40 ml ethanol)

NaCl 20 mM
Tris-HCl 2 mM (pH 7.5)

**Elution Buffer**

Tris-HCl 10 mM

pH 8.5
I.11 DNA Labelling and Detection

DIG DNA Labelling and Detection Kit that supplied by Roche was used to label DNA. Details of the content of each solution are as follows:

**Hexanucleotide Mix**

10 x concentrated hexanucleotide mixture containing:

- Random hexanucleotides: 62.5 A₂₆₀ units/ml (1.56 mg/ml)
- Tris-HCl: 500 mM
- MgCl₂: 100 mM
- Dithioerythritol (DTE): 1 mM
- BSA: 2 mg/ml
- pH 7.2

**dNTP Labelling mixture**

10 x concentrated dNTP labelling mixture containing:

- dATP: 1 mM
- dGTP: 1 mM
- dCTP: 1 mM
- dTTP: 0.65 mM
- DIG-11-dUTP: 0.35 mM
- pH 7.5

**Klenow enzyme, labelling grade**

Klenow enzyme (2U/µl)

**Anti-Digoxigenin-AP Conjugate**

Polyclonal sheep anti-digoxigenin

Fab-fragment, conjugated to alkaline phosphatase 750 U/ml

**NBT/BCIP**

10 x concentrated stock solution

**Unlabelled control DNA 1**

pBR328 (100 µg/ml) 20 µl

pBR328 is digested separately with *Bam* HI, *Bgl* I and *Hinf* I

**Unlabelled control DNA 2**

pBR328 (200 µg/ml) 20 µl

Linearised with *Bam* HI
Labelled control DNA
pBR328 DNA (5 µg/ml) 50 µl
Linearised with Bam HI

DNA Dilution Buffer
Herring sperm DNA (50 µg/ml) in 10 mM Tris-HCl,
EDTA 1mM
pH 8.0 at 20 °C

20x SSC
Per litter of molecular grade water (it was diluted to 10x, 5x, 2x, or 0.5x strength as required with sterile molecular grade water
Sodium chloride 175.32 g
Sodium citrate 88.23g
pH 7

DIG wash and Block Buffer Set (Roche)
Washing Buffer
10x stock and 1x working solution were prepared with sterile molecular grade water
Maleic acid 0.1 M
NaCl 0.15 M
Tween 20, 0.3% (v/v)
pH 7.5

Blocking solution
10x stock and 1x working solution were prepared with malic acid buffer

Detection buffer
Tris-HCl 0.1 M
NaCl 0.1 M
pH 9.5

Malic acid buffer
It was used for dilution of blocking reagent
Maleic acid 0.1 M
NaCl 0.15 M
Adjusted with NaOH to pH 7.5
I.12 Molecular weight marker

1.12.1 ColorBurst™ Electrophoresis Marker molecular weight 8 kDa-220 kDa

3µl      5µ      7µl

220 kDa
100 kDa
60 kDa
45 kDa
30 kDa
20 kDa
12 kDa
8 kDa

4-20% Tris-Glycine
1.12.2 100 bp DNA ladder

100 bp DNA ladder (Invitrogen) (0.5 µg/lane) ranged from 100-1500 bp in 100bp increments, plus a 2072 bp fragment. The 600 bp band is 2-3 times brighter than the others.

1.12.3 1kb Ladder 75-12,216 bp Gibco (Invitrogen) (0.5 µg/lane) 1 kb Ladder ranged from 75bp to 12,216 bp in various increments.
1.13 Suppliers

Bio-Rad Laboratories Ltd.
Bio-Rad House
Maxted Road
Hemel Hempstead
Hertfordshire HP2 7DX
Tel: 0800 181134
www.bio-rad.com

fisher Scientific UK Ltd
Bishop Meadow Road, Loughborough,
Leicestershire LE11 5RG
http://www.fisher.co.uk

Invitrogen / Gibco
Invitrogen Ltd
Fountain Drive Inchinnan Business Park
Paisley
PA4 9RF
www.Invitrogen.com

MWG Biotech
Eurofine MWG Operon
318 Worp Road Raynes Park
London
SW20 8QU
http://www.mwg-biotech.com

Oxoid Limited
Wade Road
Basingstoke
Hampshire
RG24 8PW
http://www.oxid.com

Qiagen Ltd.
Qiagen House
Fleming Way
Crawley
West Sussex
RH10 9NQ
www1.qiagen.com

Roche Molecular Biochemicals
Bell Lane
Lewes
East Sussex
BN7 1LG
www.biochem.roche.com

Sigma-Aldrich Company Ltd
Fancy Rd
Poole
Dorset
BH12 4QH
www.sigmaaldrich.com

Thermo Fisher Scientific
ABgene House
Blenheim Road
Epsom
Surrey
KT19 9AP
www.thermofisher.com
APPENDIX II

ADDITIONAL DATA AND IMAGES
Appendix II: Additional Data and Images

II.1 Quantification of genomic DNA extracted from *V. parahaemolyticus*

II.1 Bacterial DNA quantification

Genomic DNA from bacteria was extracted and quantified by comparison with lambda DNA standards on agarose gels (0.8%) (Section 3.8). The DNA concentration was determined by comparison with a range of the following known amounts of standard bacteriophage λ: 0.552 µg, 0.276 µg and 0.138 µg and using ‘UV Photo’ software (UVi-Tech). Alternatively DNA was quantified with a NanoDrop 100 spectrophotometer. Optical density was measured at 260 and 280 nm.

**Figure II.1 Quantification of DNA extracted from *V. parahaemolyticus* cultures.**

**Panel a:** lanes 1-10 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate E20, 5; DNA from isolate E1, 6; DNA from isolate E13, 7; DNA from isolate R1, 8; DNA from isolate C1, 9; DNA from isolate C4, 10; DNA from isolate C3. Lane M: 1kb ladders (0.3 µg).

**Panel b:** lanes 1-9 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate E10, 5; DNA from isolate C2, 6; DNA from isolate E26, 7; DNA from isolate E23, 8; DNA from isolate R2, 9; DNA from isolate E6. Lane M: 1kb ladders (0.3 µg).

**Panel c:** lanes 1-10 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate E12, 5; DNA from isolate E14, 6; DNA from isolate E15, 7; DNA from isolate E3, 8; DNA from isolate E4, 9; DNA from isolate E16, 10; DNA from isolate E22. Lane M: 1kb ladders (0.3 µg).
Figure II.1: Cont.

Panel d: lanes 1-10 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate E2, 5; DNA from isolate E5, 6; DNA from isolate E7, 7; DNA from isolate E8, 8; DNA from isolate E9, 9; DNA from isolate C5, 10; DNA from isolate C6. Lane M: 1kb ladders (0.3 µg).

Panel e: lanes 1-9 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate E11, 5; DNA from isolate E17, 6; DNA from isolate E18, 7; DNA from isolate C7, 8; DNA from isolate C8, 9; DNA from isolate C9, DNA from isolate C10. Lane M: 1kb ladders (0.3 µg).

Panel f: lanes 1-10 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate E19, 5; DNA from isolate E21, 6; DNA from isolate C11, 7; DNA from isolate C12, 8; DNA from isolate C13. Lane M: 1kb ladders (0.3 µg).

Panel g: lanes 1-10 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate E24, 5; DNA from isolate E25, 6; DNA from isolate E27, 7; DNA from isolate C14, 8; DNA from isolate C15, 9; DNA from isolate C16, 10; DNA from isolate C17. Lane M: 1kb ladders (0.3 µg).

Panel h: lanes 1-12 correspond to the following, 1; DNA from isolate E28, 2; 0.552 µg of λ standard DNA, 3; 0.276 µg of λ standard DNA, 4; 0.138 µg of λ standard DNA, 5; DNA from isolate E29, 6; DNA from isolate E30, 7; DNA from isolate E31, 8; DNA from isolate C18, 9; DNA from isolate C19, 10; DNA from isolate C20, 11; DNA from isolate C21, 12; DNA from isolate C22. Lane M: 1kb ladders (0.3 µg).
Figure II.2 Quantification of DNA extracted from *Vibrio* spp. cultures.

**Panel a:** lanes 1-10 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate VC, 5; DNA from isolate VM, 6; DNA from isolate VV1, 7; DNA from isolate VV2, 8; DNA from isolate VV3, 9; DNA from isolate VV4, 10; DNA from isolate VV5. Lane M: 1kb ladders (0.3 µg).

**Panel b:** lanes 1-9 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate VV6, 5; DNA from isolate VA1, 6; DNA from isolate VA2, 7; DNA from isolate VA3, 8; DNA from isolate VA4, 9; DNA from isolate VA5. Lane M: 1kb ladders (0.3 µg).

**Panel c:** lanes 1-12 correspond to the following, 1; 0.552 µg of λ standard DNA, 2; 0.276 µg of λ standard DNA, 3; 0.138 µg of λ standard DNA, 4; DNA from isolate VA6, 5; DNA from isolate VH1, 6; DNA from isolate VH2, 7; DNA from isolate VH3, 8; DNA from isolate VH4, 9; DNA from isolate VH5, 10; DNA from isolate VN1, 11; DNA from isolate VN2, 12; DNA from isolate VS. Lane M: 1kb ladders (0.3 µg).
Figure II.3 Quantification of DNA extracted from non-\textit{Vibrio} spp. cultures.

\textbf{Panel a}: lanes 1-9 correspond to the following, 1; 0.552 \textmu g of \textlambda standard DNA, 2; 0.276 \textmu g of \textlambda standard DNA, 3; 0.138 \textmu g of \textlambda standard DNA, 4; DNA from isolate Ah1, 5; DNA from isolate Ah2, 6; DNA from isolate As1, 7; DNA from isolate As2, 8; DNA from isolate Pf1, 9; DNA from isolate Pf2, 10. Lane M: 1kb ladders (0.3 \textmu g).  
\textbf{Panel b}: lanes 1-9 correspond to the following, 1; 0.552 \textmu g of \textlambda standard DNA, 2; 0.276 \textmu g of \textlambda standard DNA, 3; 0.138 \textmu g of \textlambda standard DNA, 4; DNA from isolate St, 5; DNA from isolate Kp, 6; DNA from isolate Ec1, 7; DNA from isolate Pv, 8; DNA from isolate Ec2, 9; DNA from isolate Sa. Lane M: 1kb ladders (0.3 \textmu g).
Table II.1 Genomic DNA concentrations determined from agarose gels (Fig. II.1). Isolates not in Fig. II.1 were quantified with a UV spectrophotometric.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>DNA conc. (ng/µl)</th>
<th>Isolate</th>
<th>DNA conc. (ng/µl)</th>
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<tbody>
<tr>
<td>R1</td>
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<td>E21</td>
<td>61.3</td>
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<td>101</td>
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<td>67</td>
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<td>VV1</td>
<td>250.9</td>
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<td>VA1</td>
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<td>VA2</td>
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<td>C21</td>
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<td>VA3</td>
<td>155.5</td>
</tr>
<tr>
<td>C22</td>
<td>90.5</td>
<td>VA4</td>
<td>134.6</td>
</tr>
<tr>
<td>R2</td>
<td>343.8</td>
<td>VA5</td>
<td>123</td>
</tr>
<tr>
<td>E1</td>
<td>53.1</td>
<td>VA6</td>
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<td>E2</td>
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<td>VH3</td>
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<td>VH5</td>
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<td>VN1</td>
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<td>As2</td>
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<td>Pf1</td>
<td>90</td>
</tr>
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<td>E15</td>
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<td>10</td>
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<td>E17</td>
<td>151.8</td>
<td>St</td>
<td>23</td>
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<td>390.8</td>
<td>Pv</td>
<td>15.9</td>
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<td>Ec1</td>
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<tr>
<td></td>
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<td>Ec2</td>
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</tr>
</tbody>
</table>
II.2 Additional gel images relevant to Chapter 4

Figure II.4 SDS-PAGE of soluble whole cell proteins from a clinical *V. parahaemolyticus* isolate (C1) with different concentrations. M, protein marker (M.W. =8 kDa to 220 kDa).

Figure II.5 SDS-PAGE of soluble whole cell proteins from an environmental *V. parahaemolyticus* isolate (E16) with different concentrations. M, protein marker (M.W. =8 kDa-220 kDa).
Figure II.6 SDS-PAGE of soluble whole cell proteins from a clinical V. parahaemolyticus isolate (C7) with different concentrations. M, protein marker (M.W. = 8 kDa-220 kDa).

Figure II.7 SDS-PAGE of soluble whole cell proteins from a clinical V. parahaemolyticus isolate (C4) with different concentrations. M, protein marker (M.W. = 8 kDa-220 kDa).
II.3 Statistical analysis relevant to Chapter 4

Chi-Square Test: KP+, KP-

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

<table>
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<th>KP+</th>
<th>KP-</th>
<th>Total</th>
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<tr>
<td></td>
<td>10.04</td>
<td>12.96</td>
<td></td>
</tr>
<tr>
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<td>8.006</td>
<td>6.198</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>27</td>
<td>32</td>
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<tr>
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Chi-Sq = 24.412, DF = 1, P-Value = 0.000

Chi-Square Test: Ure+, Ure-

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

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<td></td>
<td>15.89</td>
<td>7.11</td>
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<tr>
<td></td>
<td>3.180</td>
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<td>17</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>22.11</td>
<td>9.89</td>
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</tr>
<tr>
<td></td>
<td>2.286</td>
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<tr>
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</tbody>
</table>

Chi-Sq = 17.685, DF = 1, P-Value = 0.000

Chi-Square Test: Lip+, Lip-

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

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<td></td>
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Chi-Sq = 0.915, DF = 1, P-Value = 0.339
1 cells with expected counts less than 5
Chi-Square Test: Pro+, Pro-

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

<table>
<thead>
<tr>
<th></th>
<th>Pro+</th>
<th>Pro-</th>
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<tr>
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<td>12.80</td>
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<tr>
<td></td>
<td>1.959</td>
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<td>1.408</td>
<td>2.112</td>
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<tr>
<td>Total</td>
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<td>22</td>
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</tbody>
</table>

Chi-Sq = 8.419, DF = 1, P-Value = 0.004

Chi-Square Test: PPL+, PPL-

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

<table>
<thead>
<tr>
<th></th>
<th>PPL+</th>
<th>PPL-</th>
<th>Total</th>
<th></th>
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</tr>
</thead>
<tbody>
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<td>8.006</td>
<td>6.198</td>
</tr>
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<td>2</td>
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<td>18.04</td>
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<td>5.754</td>
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<td>8.006</td>
<td>6.198</td>
<td></td>
<td>5.754</td>
<td>4.455</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>31</td>
<td>55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chi-Sq = 24.412, DF = 1, P-Value = 0.000

Chi-Square Test: tdh+, tdh-

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

<table>
<thead>
<tr>
<th></th>
<th>tdh+</th>
<th>tdh-</th>
<th>Total</th>
<th></th>
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<td>4.072</td>
<td>2.927</td>
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<tr>
<td>Total</td>
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<td>32</td>
<td>55</td>
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</tbody>
</table>

Chi-Sq = 16.736, DF = 1, P-Value = 0.000

Chi-Square Test: trh+, trh-

Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

<table>
<thead>
<tr>
<th></th>
<th>trh+</th>
<th>trh-</th>
<th>Total</th>
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<td>0.013</td>
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<tr>
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<td>0.058</td>
<td>0.018</td>
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<td>0.042</td>
<td>0.013</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>42</td>
<td>55</td>
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<td></td>
</tr>
</tbody>
</table>

Chi-Sq = 0.132, DF = 1, P-Value = 0.717
Correlations: KP, tdh

Pearson correlation of KP and tdh = 0.741
P-Value = 0.000

Correlations: Ure, trh

Pearson correlation of Ure and trh = 0.280
P-Value = 0.03

Correlations: plasmid, KP

Pearson correlation of KP and plasmid = 0.011
P-Value = 0.934

Correlations: plasmid, tdh

Pearson correlation of tdh and plasmid = -0.120
P-Value = 0.383

Correlations: plasmid, Ure

Pearson correlation of Ure and plasmid = -0.120
P-Value = 0.381

Correlations: plasmid, trh

Pearson correlation of trh and plasmid = -0.206
P-Value = 0.132

II.3 Statistical analysis relevant to Chapter 5

Two-Sample Comparison - Caco Clin & Caco Env

Comparison of Means

95.0% confidence interval for mean of Caco Clin: 69.5381 +/- 3.42343 [66.1147, 72.9615]
95.0% confidence interval for mean of Caco Env: 17.4866 +/- 3.91195 [13.5746, 21.3985]
95.0% confidence interval for the difference between the means assuming equal variances: 52.0516 +/- 5.41566 [46.6359, 57.4672]

t test to compare means
Null hypothesis: mean1 = mean2
Alt. hypothesis: mean1 NE mean2
assuming equal variances: t = 18.9787  P-value = 0
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 46.6359 to 57.4672. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.
Comparison of Medians
Median of sample 1: 68.48
Median of sample 2: 18.18

Mann-Whitney (Wilcoxon) W-test to compare medians
Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

Average rank of sample 1: 127.464
Average rank of sample 2: 51.0417

W = 244.0  P-value = 0
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

Two-Sample Comparison - Caco KP+ & Caco KP-

Comparison of Means

95.0% confidence interval for mean of Caco KP+: 64.7686 +/- 5.12078  [59.6478, 69.8894]
95.0% confidence interval for mean of Caco KP-: 19.6483 +/- 4.41773  [15.2306, 24.066]
95.0% confidence interval for the difference between the means
assuming equal variances: 45.1203 +/- 6.6953  [38.425, 51.8156]

A t-test to compare means
Null hypothesis: mean1 = mean2
Alt. hypothesis: mean1 NE mean2
   assuming equal variances: t = 13.3072  P-value = 0
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 38.425 to 51.8156. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

Comparison of Medians
Median of sample 1: 67.895
Median of sample 2: 18.48

Mann-Whitney (Wilcoxon) W-test to compare medians
Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

Average rank of sample 1: 121.229
Average rank of sample 2: 53.4032

W = 595.5  P-value = 0
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.
Two-Sample Comparison - Caco UR+ & Caco UR-

Comparison of Means
95.0% confidence interval for mean of Caco UR+: 51.7708 +/- 4.78097 [46.9898, 56.5518]
95.0% confidence interval for mean of Caco UR-: 11.2739 +/- 6.31226 [4.96166, 17.5862]
95.0% confidence interval for the difference between the means assuming equal variances: 40.4969 +/- 8.24776 [32.2491, 48.7446]

t test to compare means
Null hypothesis: mean1 = mean2
Alt. hypothesis: mean1 NE mean2
assuming equal variances: t = 9.69551  P-value = 2.24304E-7
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 32.2491 to 48.7446. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

Comparison of Medians
Median of sample 1: 60.45
Median of sample 2: 1.24

Mann-Whitney (Wilcoxon) W-test to compare medians
Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2
Average rank of sample 1: 103.044
Average rank of sample 2: 38.1961
W = 622.0  P-value = 0
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

Two-Sample Comparison - Caco PPL+ & Caco PPL-

Comparison of Means
95.0% confidence interval for mean of Caco PPL+: 66.8646 +/- 4.64112 [62.2235, 71.5057]
95.0% confidence interval for mean of Caco PPL-: 17.8773 +/- 3.89509 [13.9822, 21.7724]
95.0% confidence interval for the difference between the means assuming equal variances: 48.9873 +/- 5.97691 [43.0104, 54.9642]

t test to compare means
Null hypothesis: mean1 = mean2
Alt. hypothesis: mean1 NE mean2
assuming equal variances: t = 16.1842  P-value = 0
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 43.0104 to 54.9642. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.
Comparison of Medians
Median of sample 1: 68.865
Median of sample 2: 17.88

Mann-Whitney (Wilcoxon) W-test to compare medians
Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

Average rank of sample 1: 124.618
Average rank of sample 2: 50.7796

W = 351.5  P-value = 0
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

Two-Sample Comparison - Caco Pro+ & Caco Pro-

Comparison of Means
95.0% confidence interval for mean of Caco Pro+: 50.6645 +/- 5.0673  [45.5972, 55.7318]
95.0% confidence interval for mean of Caco Pro-: 21.4126 +/- 7.31562  [14.097, 28.7282]
95.0% confidence interval for the difference between the means
assuming equal variances: 29.252 +/- 8.54313  [20.7088, 37.7951]

t test to compare means
Null hypothesis: mean1 = mean2
Alt. hypothesis: mean1 NE mean2
assuming equal variances: t = 6.76119  P-value = 1.81643E-7
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 20.7088 to 37.7951. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

Comparison of Medians
Median of sample 1: 57.27
Median of sample 2: 3.755

Mann-Whitney (Wilcoxon) W-test to compare medians
Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

Average rank of sample 1: 102.475
Average rank of sample 2: 53.7879

W = 1339.0  P-value = 1.43423E-10
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.
Two-Sample Comparison - Caco Lip+ & Caco Lip-

Comparison of Means
95.0% confidence interval for mean of Caco Lip+: 47.6503 +/- 10.958 [36.6923, 58.6083]
95.0% confidence interval for mean of Caco Lip-: 37.1544 +/- 5.30858 [31.8458, 42.463]
95.0% confidence interval for the difference between the means
assuming equal variances: 10.4959 +/- 11.8541 [-1.35824, 22.3501]

t test to compare means
Null hypothesis: mean1 = mean2
Alt. hypothesis: mean1 NE mean2
assuming equal variances: t = 1.74838  P-value = 0.0822816
Do not reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from -1.35824 to 22.3501. Since the interval contains the value 0, there is not a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is not less than 0.05, we cannot reject the null hypothesis.

Comparison of Medians
Median of sample 1: 46.97
Median of sample 2: 27.73

Mann-Whitney (Wilcoxon) W-test to compare medians
Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

Average rank of sample 1: 96.8485
Average rank of sample 2: 79.5379

W = 1721.0  P-value = 0.0628648
Do not reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is greater than or equal to 0.05, there is not a statistically significant difference between the medians at the 95.0% confidence level.

Two-Sample Comparison - Caco tdh+ & Caco tdh-

Comparison of Means
95.0% confidence interval for mean of Caco tdh+: 62.3517 +/- 6.18497 [56.1668, 68.5367]
95.0% confidence interval for mean of Caco tdh-: 22.6518 +/- 4.63173 [18.02, 27.2835]
95.0% confidence interval for the difference between the means
assuming equal variances: 39.7 +/- 7.51257 [32.1874, 47.2125]

t test to compare means
Null hypothesis: mean1 = mean2
Alt. hypothesis: mean1 NE mean2
assuming equal variances: t = 10.4349  P-value = 3.53133E-7
Reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 32.1874 to 47.2125. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.
A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

**Comparison of Medians**

<table>
<thead>
<tr>
<th>Median of sample 1</th>
<th>68.12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median of sample 2</td>
<td>19.86</td>
</tr>
</tbody>
</table>

Mann-Whitney (Wilcoxon) W-test to compare medians

<table>
<thead>
<tr>
<th>Null hypothesis: median1 = median2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alt. hypothesis: median1 NE median2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average rank of sample 1</th>
<th>117.833</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average rank of sample 2</td>
<td>57.9635</td>
</tr>
</tbody>
</table>

W = 908.5  P-value = 0

Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples

**Comparison of Means**

95.0% confidence interval for mean of Caco trh+: 47.3079 +/- 8.90681  [38.4011, 56.2148]
95.0% confidence interval for mean of Caco trh-: 36.7606 +/- 5.59758  [31.163, 42.3581]
95.0% confidence interval for the difference between the means assuming equal variances: 10.5474 +/- 11.1463  [-0.598866, 21.6937]

t-test to compare means

<table>
<thead>
<tr>
<th>Null hypothesis: mean1 = mean2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alt. hypothesis: mean1 NE mean2</td>
</tr>
<tr>
<td>assuming equal variances: t = 1.86854  P-value = 0.0634821</td>
</tr>
</tbody>
</table>

Do not reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from -0.598866 to 21.6937. Since the interval contains the value 0, there is not a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is not less than 0.05, we cannot reject the null hypothesis.

**Comparison of Medians**

<table>
<thead>
<tr>
<th>Median of sample 1</th>
<th>46.97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median of sample 2</td>
<td>27.515</td>
</tr>
</tbody>
</table>

Mann-Whitney (Wilcoxon) W-test to compare medians

<table>
<thead>
<tr>
<th>Null hypothesis: median1 = median2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alt. hypothesis: median1 NE median2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average rank of sample 1</th>
<th>97.0897</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average rank of sample 2</td>
<td>78.6389</td>
</tr>
</tbody>
</table>

W = 1907.5  P-value = 0.0351847

Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples.
in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

**Two-Sample Comparison - CHO Clin & CHO Env**

**Comparison of Means**
- 95.0% confidence interval for mean of CHO Clin: 59.6314 +/- 1.94802  [57.6834, 61.5795]
- 95.0% confidence interval for mean of CHO Env: 12.1842 +/- 2.84031  [9.34386, 15.0245]
- 95.0% confidence interval for the difference between the means assuming equal variances: 47.4473 +/- 3.7132  [43.7341, 51.1605]

**t test to compare means**
- Null hypothesis: mean1 = mean2
- Alt. hypothesis: mean1 NE mean2
  - assuming equal variances: t = 25.2318  P-value = 0
- Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 43.7341 to 51.1605. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

**Comparison of Medians**
- Median of sample 1: 61.21
- Median of sample 2: 5.76

**Mann-Whitney (Wilcoxon) W-test to compare medians**
- Null hypothesis: median1 = median2
- Alt. hypothesis: median1 NE median2
- Average rank of sample 1: 129.094
- Average rank of sample 2: 49.8698
- W = 131.5  P-value = 0
- Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**
This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

**Two-Sample Comparison - CHO KP+ & CHO KP-**

**Comparison of Means**
- 95.0% confidence interval for mean of CHO KP+: 53.7301 +/- 4.37219  [49.3579, 58.1023]
- 95.0% confidence interval for mean of CHO KP-: 15.2224 +/- 3.64138  [11.581, 18.8637]
- 95.0% confidence interval for the difference between the means assuming equal variances: 38.5078 +/- 5.60727  [32.9005, 44.115]

**t test to compare means**
- Null hypothesis: mean1 = mean2
- Alt. hypothesis: mean1 NE mean2
  - assuming equal variances: t = 13.5607  P-value = 0
- Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**
This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 32.9005 to 44.115. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.
A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

**Comparison of Medians**

Median of sample 1: 61.21
Median of sample 2: 5.76

Mann-Whitney (Wilcoxon) W-test to compare medians

Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

Average rank of sample 1: 120.535
Average rank of sample 2: 53.9409

W = 645.5   P-value = 0
Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

**Two-Sample Comparison - CHO PPL+ & CHO PPL-**

**Comparison of Means**

95.0% confidence interval for mean of CHO PPL+: 55.8318 +/- 3.65356   [52.1782, 59.4854]
95.0% confidence interval for mean of CHO PPL-: 13.5953 +/- 3.32348   [10.2718, 16.9187]
95.0% confidence interval for the difference between the means assuming equal variances: 42.2365 +/- 4.92335   [37.3132, 47.1599]

**t-test to compare means**

Null hypothesis: mean1 = mean2
Alt. hypothesis: mean1 NE mean2

assuming equal variances: t = 16.94   P-value = 0
Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 37.3132 to 47.1599. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

**Comparison of Medians**

Median of sample 1: 61.255
Median of sample 2: 5.76

Mann-Whitney (Wilcoxon) W-test to compare medians

Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

Average rank of sample 1: 125.271
Average rank of sample 2: 50.2742

W = 304.5   P-value = 0
Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.
Two-Sample Comparison - CHO Pro+ & CHO Pro-

Comparison of Means
95.0% confidence interval for mean of CHO Pro+: 40.8176 +/- 4.76495 [36.0526, 45.5825]
95.0% confidence interval for mean of CHO Pro-: 18.838 +/- 5.97901 [12.859, 24.817]
95.0% confidence interval for the difference between the means

**t test to compare means**

Null hypothesis: mean1 = mean2  
Alt. hypothesis: mean1 NE mean2
  assuming equal variances: t = 5.74787  P-value = 2.18783E-7  
  Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a **t-test** to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 14.4287 to 29.5304. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A **t-test** may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

Comparison of Medians
Median of sample 1: 49.39  
Median of sample 2: 5.76

**Mann-Whitney (Wilcoxon) W-test to compare medians**
  Null hypothesis: median1 = median2  
  Alt. hypothesis: median1 NE median2
  Average rank of sample 1: 98.5101
  Average rank of sample 2: 59.7348
  W = 1731.5  P-value = 3.28365E-7  
  Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a **Mann-Whitney W-test** to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

Two-Sample Comparison - CHO UR + & CHO UR –

Comparison of Means
95.0% confidence interval for mean of CHO UR +: 41.6224 +/- 4.52674 [37.0956, 46.1491]
95.0% confidence interval for mean of CHO UR -: 11.1151 +/- 4.41331 [6.70178, 15.5284]
95.0% confidence interval for the difference between the means
  assuming equal variances: 30.5073 +/- 7.3494 [23.1579, 37.8567]

**t test to compare means**

Null hypothesis: mean1 = mean2  
Alt. hypothesis: mean1 NE mean2
  assuming equal variances: t = 8.19666  P-value = 2.28026E-7  
  Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a **t-test** to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 23.1579 to 37.8567. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A **t-test** may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the
two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

**Comparison of Medians**

Median of sample 1: 50.91  
Median of sample 2: 4.55  

Mann-Whitney (Wilcoxon) W-test to compare medians  
Null hypothesis: median1 = median2  
Alt. hypothesis: median1 NE median2  

Average rank of sample 1: 100.75  
Average rank of sample 2: 43.3235  

W = 883.5  P-value = 0  
Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

**Two-Sample Comparison - CHO Lip+ & CHO Lip-**

**Comparison of Means**

95.0% confidence interval for mean of CHO Lip+: 39.2352 +/- 9.40938  [29.8258, 48.6445]  
95.0% confidence interval for mean of CHO Lip-: 30.2234 +/- 4.48393  [25.7395, 34.7073]  
95.0% confidence interval for the difference between the means  
assuming equal variances: 9.01174 +/- 10.0457  [-1.03392, 19.0574]

* t-test to compare means  
Null hypothesis: mean1 = mean2  
Alt. hypothesis: mean1 NE mean2  
assuming equal variances: t = 1.7714  P-value = 0.0783638  
Do not reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a t-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from -1.03392 to 19.0574. Since the interval contains the value 0, there is not a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A t-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is not less than 0.05, we cannot reject the null hypothesis.

**Comparison of Medians**

Median of sample 1: 49.7  
Median of sample 2: 24.545  

Mann-Whitney (Wilcoxon) W-test to compare medians  
Null hypothesis: median1 = median2  
Alt. hypothesis: median1 NE median2  

Average rank of sample 1: 100.394  
Average rank of sample 2: 78.6515  

W = 1604.0  P-value = 0.0294519  
Reject the null hypothesis for alpha = 0.05.

**The StatAdvisor**

This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.
Two-Sample Comparison - CHO tdh+ & CHO tdh-

Comparison of Means
95.0% confidence interval for mean of CHO tdh+: 51.0893 +/- 5.0786 [46.0107, 56.1679]
95.0% confidence interval for mean of CHO tdh-: 18.3239 +/- 4.159 [14.1649, 22.4829]
95.0% confidence interval for the difference between the means

\begin{align*}
& t \text{ test to compare means} \\
& \quad \text{Null hypothesis: mean1 = mean2} \\
& \quad \text{Alt. hypothesis: mean1 NE mean2} \\
& \quad \text{assuming equal variances: } t = 9.98821 \quad \text{P-value } = 2.6116 \times 10^{-7} \\
& \text{Reject the null hypothesis for alpha } = 0.05.
\end{align*}

The StatAdvisor
This option runs a \( t \)-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from 26.2878 to 39.243. Since the interval does not contain the value 0, there is a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A \( t \)-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is less than 0.05, we can reject the null hypothesis in favor of the alternative.

Comparison of Medians
Median of sample 1: 60.91
Median of sample 2: 5.76

Mann-Whitney (Wilcoxon) \( W \)-test to compare medians
Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

\begin{align*}
& \text{Average rank of sample 1: 116.848} \\
& \text{Average rank of sample 2: 58.6719} \\
& W = 976.5 \quad \text{P-value } = 0 \\
& \text{Reject the null hypothesis for alpha } = 0.05.
\end{align*}

The StatAdvisor
This option runs a Mann-Whitney \( W \)-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level.

Two-Sample Comparison - CHO trh+ & CHO trh-

Comparison of Means
95.0% confidence interval for mean of CHO trh+: 36.5133 +/- 8.0465 [28.4668, 44.5599]
95.0% confidence interval for mean of CHO trh-: 30.6367 +/- 4.7066 [25.9301, 35.3434]
95.0% confidence interval for the difference between the means
assuming equal variances: 5.87659 +/- 9.50537 [-3.62879, 15.382]

\begin{align*}
& t \text{ test to compare means} \\
& \quad \text{Null hypothesis: mean1 = mean2} \\
& \quad \text{Alt. hypothesis: mean1 NE mean2} \\
& \quad \text{assuming equal variances: } t = 1.22079 \quad \text{P-value } = 0.223928 \\
& \text{Do not reject the null hypothesis for alpha } = 0.05.
\end{align*}

The StatAdvisor
This option runs a \( t \)-test to compare the means of the two samples. It also constructs confidence intervals or bounds for each mean and for the difference between the means. Of particular interest is the confidence interval for the difference between the means, which extends from -3.62879 to 15.382. Since the interval contains the value 0, there is not a statistically significant difference between the means of the two samples at the 95.0% confidence level.

A \( t \)-test may also be used to test a specific hypothesis about the difference between the means of the populations from which the two samples come. In this case, the test has been constructed to determine whether the difference between the two means equals 0.0 versus the alternative hypothesis that the difference does not equal 0.0. Since the computed P-value is not less than 0.05, we cannot reject the null hypothesis.

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Comparison of Medians
Median of sample 1: 48.24
Median of sample 2: 20.91

Mann-Whitney (Wilcoxon) W-test to compare medians
Null hypothesis: median1 = median2
Alt. hypothesis: median1 NE median2

Average rank of sample 1: 90.3846
Average rank of sample 2: 80.7143

W = 2169.0   P-value = 0.270061
Do not reject the null hypothesis for alpha = 0.05.

The StatAdvisor
This option runs a Mann-Whitney W-test to compare the medians of the two samples. This test is constructed by combining the two samples, sorting the data from smallest to largest, and comparing the average ranks of the two samples in the combined data. Since the P-value is greater than or equal to 0.05, there is not a statistically significant difference between the medians at the 95.0% confidence level.
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