CHARACTERISATION AND MOLECULAR TYPING OF CLINICAL AND ENVIRONMENTAL ISOLATES OF *VIBRIO PARAHAEOLYTICUS*

by

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This thesis is dedicated to
my mother, T. N. Khatoon and
my late grandfather, A. Talukdar
Abstract

Characterisation and Molecular Typing of Clinical and Environmental Isolates of Vibrio parahaemolyticus

Aklak Miah

Vibrio parahaemolyticus is a natural inhabitant of coastal waters worldwide and is the leading cause of seafood-borne gastroenteritis. This study reports on the use of several molecular characterisation methods to screen clinical and environmental isolates of V. parahaemolyticus to assess whether such techniques can be used to distinguish pathogenic isolates reliably.

In a total of 86 isolates mainly of V. parahaemolyticus but also including V. cholerae, V. vulnificus and several other species, serotypes of the more virulent clonal group O3:K6 were identified, but otherwise there appeared no association with serotype and phenotype. The tdh and trh genes encoding haemolysins that are typically associated with virulent isolates were found in a significantly large number of isolates; however, poor concordance between haemolytic activity and the presence of the gene tdh was found. In an effort to establish more accurate relationships amongst clinical and environmental isolates of V. parahaemolyticus, four molecular typing systems were employed; namely pulsed-field gel electrophoresis (PFGE), intergenic transcribed spacer (ITS) analysis, tDNA intergenic length polymorphisms (tDNA-ILPs) and randomly amplified polymorphic DNA (RAPD). Typing patterns and clustering analysis using these methods differentiated V. parahaemolyticus from other marine species as well as at the subspecies level. PFGE with NotI was shown to be the most discriminative but suffered from not being universally applicable. Both ITS and tDNA-ILP methods were sufficiently discriminatory with discrimination indices (DI) of between 0.568 and 0.724, depending on the primers employed. The discriminatory ability of RAPD was also affected by the primers used (DI = 0.959 - 0.965) but closely matched that of PFGE (DI = 0.976). Additionally, both RAPD methods were able to distinguish putative markers for the pandemic clonal group. Typing systems appeared largely stable in duplicate and triplicate analyses with multiple primer pairs with some obvious variability in the reproduction of faint amplicons. All methods except PFGE were simple to execute but none of the methods could distinguish V. parahaemolyticus into obvious lineages based on the clinical or environmental source.

With the recent implication of a type III secretion system (TTSS) involved in the pathogenicity of V. parahaemolyticus, a multiplex PCR system using PCR primers that spanned both TTSS1 and TTSS2 regions was developed. Dot-blot analysis confirmed TTSS2 genes in at least 30% of environmental isolates. Nucleotide sequence analysis revealed 100% sequence homology in three loci of TTSS2 putative structural genes. In comparison, a total of 34 single nucleotide polymorphisms (SNP) were identified in three TTSS1 regions. In two of the regions, the SNPs were synonymous, whereas a non-synonymous substitution in the structural gene vcrD resulted in valine replacement with isoleucine. In addition, nucleotide deletions in TTSS1 with resultant frameshift mutations were identified. The finding that significant numbers of environmental isolates also possess TTSS2 genes is contrary to currently held opinion that TTSS2 is only present in clinical isolates. It is hypothesised that the high incidences of V. parahaemolyticus infections may be related to active TTSS2 genes, whereas a high degree of polymorphisms in TTSS1 suggest it may be inactive.
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<td>Adenine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CEFAS</td>
<td>Centre for Environment Fisheries and Aquaculture Science</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CHEF</td>
<td>contour-clamped homogenous field</td>
</tr>
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<td>CSS</td>
<td>cell suspension solution</td>
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<td>tris-borate EDTA buffer</td>
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<td>Thiosulphate Citrate Bile Salt agar medium</td>
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<td>thermostable direct haemolysin gene</td>
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<td>TE</td>
<td>Tris-ethylenediaminetetraacetic acid</td>
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<td>thermolabile haemolysin gene</td>
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<td>thermostable direct haemolysin related gene</td>
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<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
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<td>transfer ribonucleic acid</td>
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<td>tryptone soya agar</td>
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<td>TSB</td>
<td>tryptone soya broth</td>
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<tr>
<td>TTSS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UPGMA</td>
<td>unweighed pair group method using arithmetic averages</td>
</tr>
<tr>
<td>ure</td>
<td>urease gene cluster for urea hydrolysis</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
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V  volts
v/v  volume per volume
w/v  weight per volume
Acknowledgements

I am most indebted to Dr Martyn Gilpin who was Director of Studies, for the continual day-to-day supervision and advice throughout the course of this study. My deep appreciations extend to my supervisors Dr Colin Munn and Dr Rachel Rangdale for their thoughts on my work and reading through my thesis. Thank you Rachel for giving me the opportunity to study for a PhD, organising the funding through CEFAS throughout the full duration of my studies and the opportunity to carry out some of the serotyping and BioNumerics data analysis in Weymouth.

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Author's signed 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.

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A programme of advanced study was undertaken, which included postgraduate research training (MSc modules EAR5101, ENV5101 and IMS5101) on research methods and skills.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes and several papers are in preparation for publication.

Publications - In Preparation

Population diversity of clinical and environmental *Vibrio parahaemolyticus* based on polymorphisms of 16S - 23S and tDNA spacer regions.

Identification of Kanagawa positive *V. parahaemolyticus* in British coastal waters and markers for the clonally related pandemic O3:K6 subpopulation.

Type III secretion system two (TTSS2) of virulent *Vibrio parahaemolyticus* is conserved and is also found distributed amongst Kanagawa negative environmental isolates and other *Vibrio* spp.

Conferences


Oral presentations


‘Vibrio parahaemolyticus – a menace of warm coastal waters’ Ecotoxicology and Stress Biology Research Seminar, School of Biological Sciences, University of Plymouth. October. 2003.

‘Pathogencity of Vibrio parahaemolyticus’ CEFAS Weymouth Laboratory, October 2005.

Other relevant conferences, courses and workshops attended

Peninsula Microbiology Forum. One day meeting sponsored by the SGM and SfAM. Marine Biological Association. March 2003

Society for General Microbiology. 152nd Meeting. Microbial Subversion of Host Cells. University of Edinburgh, 7-11th April 2003

Society for General Microbiology. 153rd Meeting. Exploiting Genomes: Bases to Megabases in 50 years. UMIST, 8-11th September 2003

Bioinformatics Workshop – Sequence Analysis and Comparison with Artemis and the Artemis Comparison Tool (ACT). Wellcome Trust Sanger Institute & the SGM, University of Bristol, August 2003

Bioinformatics Summer School – BCS & School of Computing, University of Exeter 30th August to 2nd September 2004


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Signed

Date 23/09/09

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Chapter One

INTRODUCTION

1.1 Introduction

*Vibrio parahaemolyticus* is a bacterium found in the marine environment. It is phylogenetically close to *V. cholerae*, the etiological agent of cholera and can cause human disease upon ingestion of seafood contaminated with the bacteria. Worldwide, *V. parahaemolyticus* is a significant cause of food borne gastroenteritis, especially in warmer coastal regions. In Japan and the USA it is the leading cause of seafood related gastroenteritis (Pan et al., 1997). In the UK and Europe, *V. parahaemolyticus* is an increasing concern due to the rise in consumption of raw or undercooked seafood and the rise of seawater temperatures. The first UK outbreak was recorded in 1974 when 12 holiday makers became ill after eating dressed crab (Hooper et al., 1974) and since then there have been several other reports of sporadic outbreaks of *V. parahaemolyticus* food poisoning in Europe (Hervio-Heath et al., 2002; Lozano-Leon et al., 2003; Marne & Aznar, 1988; Robert-Pillot et al., 2004). Although most harvested fish and shellfish, especially those from brackish, estuarine and coastal environments contain *V. parahaemolyticus*, only a small proportion of isolates are thought to be pathogenic (Cook et al., 2002; DePaola et al., 2003a; Deepanjali et al., 2005).

For many years the isolation and distinction of *V. parahaemolyticus* from the closely related *Vibrio* species that inhabit marine and estuarine habitats, as well as from contaminated seafood, have proved to be arduous and long. The detection of virulent *V. parahaemolyticus* amongst a numerically greater number of avirulent *V. parahaemolyticus* thus poses an even greater problem. To date, there appears to be no widely accepted method for the enumeration of virulent isolates with increasing uncertainties of using the
total *V. parahaemolyticus* count to predict the risk of infection (Zimmerman et al., 2007). These methods of *V. parahaemolyticus* detection usually require several days using conventional techniques (De Sousa et al., 2004). The approach is based on presumptive incubation in a non-selective broth followed by confirmatory growth on selective agar and biochemical and nutritional tests. This process together with a lack of distinct phenotype to ascertain pathogenic isolates and the inherent nature of a self-limiting gastroenteritis infection means that most cases of infection either remain unreported or the pathogen is not detected. It is usually only in severe cases requiring hospitalisation that tests for pathogenic *V. parahaemolyticus* can be carried out, hence, adequate surveillance methods have long been overdue for this organism.

In recent years a number of molecular methods have been developed to aid the process of detection, identification and enumeration of potentially pathogenic *V. parahaemolyticus* using polymerase chain reaction (PCR) based approaches (Kim et al., 1999; Miwa et al., 2003; Panicker et al., 2004). These include the use of species specific and recognised virulence gene markers, and in many cases multiple gene targets are detected in multiplex PCR reactions (Bej et al., 1999). However, due to a lack of understanding of what constitutes a true pathogenic strain of *V. parahaemolyticus*, there are no satisfactory detection methods to identify pathogenic isolates. Traditional typing systems such as biochemical and nutritional profiling and O and K antigenic serotyping, although useful in identifying certain pandemic clones, have failed to provide a reliable means for discriminating strains that are pathogenic, due to a remarkable degree of phenotypic and serological uniformity (Bhuiyan et al., 2002; Binta & Nyaga, 1982; Iguchi et al., 1995; Martinez-Urtaza et al., 2006; Rahman et al., 2006). Thus, an increasing number of single and multiple molecular characterisation systems are currently being developed and assessed, to enable reliable, reproducible and robust analysis of genetic diversity amongst virulent and avirulent strains. These include pulsed field gel electrophoresis (PFGE)
methods (Wong et al., 2007), various PCR methods (Ward & Bej, 2006) and the increasing use of genomic sequencing data for applications such as mapping intergenic spacer regions (Gonzalez-Escalona et al., 2006a) and multilocus sequence typing (MLST) González-Escalona et al., 2008)

The use of a variety of molecular subtyping systems almost always generates divergent phylogenetic clusters as each typing system works by utilising different loci or traits. Therefore the dependence on a few properties or just one typing system may lead to errors that may not be apparent whilst elucidating relatedness amongst isolates (Maslow & Mulligan, 1996). For example, clonally related pathogenic strains of *V. parahaemolyticus* may show identical PFGE profiles with respect to the restriction fragment produced but fall into two distinct serovars (Chowdhury et al. 2000a). Hence another enzyme or second method must be employed to validate the degree of relatedness. Generally, at least two reproducible and reliable typing systems should be used, together with further characterisation of specific markers of variation in order to confirm epidemiological relatedness between isolates.

All bacterial molecular typing systems are based upon the principle that epidemiologically related isolates are derivatives of a single parent isolate (Struelens, 1998). Therefore the pathogenic isolate shares characteristics that may differ from those of non-pathogenic isolates within the same species. Thus typing systems are able to discriminate between epidemiologically related and non-related isolates, although typing methods are more frequently used to characterise organisms below the species level (Kostman, 1995).

1.2 Aims and Objectives

Despite the large progress in identification and detection of *V. parahaemolyticus*, only two major virulence genes encoding for thermostable haemolysins have been used in detection
protocols having been found in 94% - 97% of clinical cases (Suzuki et al., 1997). These genes are rarely detected from environmental and food samples so the significance of finding environmental reservoirs of haemolysin genes remains unclear.

Although various serovars of *V. parahaemolyticus* can cause infections, to date there is no method of biochemically, serotypically or phenotypically differentiating clinical (virulent) and environmental (mostly avirulent) isolates of this organism. Current molecular typing systems relate pathogenic isolates or outbreaks of disease to epidemiological purposes and clonal relatedness yet there are no standardised, robust methods in use and as such considerable variability in data and its interpretation seriously hamper characterising virulent isolates. This, together with inadequate means to differentiate clinical and environmental isolates further exacerbates the problem. The overall aim of the work presented in this thesis was to assess the use of molecular characterisation to differentiate clinical and environmental isolates of *V. parahaemolyticus*.

The specific objectives were:

i) to assess existing biochemical and molecular typing systems in the specific characterisation and differentiation of *V. parahaemolyticus* using phenotypic and genotypic characteristics including growth, haemolysis and subtyping systems of PFGE and 16S-23S intergenic spacer analysis (chapters 4, 5 & 6)

ii) to develop previously described typing systems for use with *V. parahaemolyticus* including ITS1, tRNA typing and RAPD, (chapters 6, 7 and 8)

iii) to use the recently identified type III secretion system (TTSS) (Makino et al., 2003; Park et al., 2004b; Ono et al., 2006) to assess differentiation between clinical and environmental isolates of *V. parahaemolyticus* (chapter 9)
finally, to compare and evaluate all methods described to differentiate clinical and environmental isolates of this pathogen (chapters 10 & 11).

A better understanding of the differences and relatedness between pathogenic and non-pathogenic *V. parahaemolyticus* enables the development of more accurate targeting, detection and monitoring of this emerging global pathogen.
2.1 The Organism

2.1.1 General characteristics

*V. parahaemolyticus* is a Gram-negative, motile, rod-shaped bacterium possessing both polar and lateral flagella that enable it to swim and swarm respectively (Atsumi *et al.*, 1992; Kim & McCarter, 2000; McCarter, 2001). The organism is generally oxidase positive, facultatively aerobic and non-spore forming. It is a member of the *Vibrionaceae* of the γ-Proteobacteria family, originally termed *Pasteurella parahaemolytica* (Fujino, 1966). In common with other vibrios, *V. parahaemolyticus* has a strong requirement for sodium ions for growth (Pace, 1989) and is abundant in the marine and estuarine environments (Baross & Liston, 1970; Larsen *et al.*, 1981; Binta & Nyaga, 1982). Together with *V. cholerae* and *V. vulnificus*, *V. parahaemolyticus* form a group of organisms that are responsible for most bacterial seafood and water-borne extra-intestinal infections (Johnson *et al.*, 1971; Thornton *et al.*, 2002). Recently, a number of advances in molecular methods have facilitated the study of the dynamics and pathogenicity of these organisms.

2.1.2 Genome organisation of *V. parahaemolyticus*

Pulsed-field gel electrophoresis (PFGE, section 2.7.3) of intact genomic DNA shows *V. parahaemolyticus* to possess two chromosomes (replicons) of approximately 3.2 and 1.9 Mb (Yamaichi *et al.*, 1999) and that the two-chromosome configuration is common amongst members of the *Vibrionaceae* (Tagomori *et al.*, 2002; Okada *et al.*, 2005). The larger replicon ranged from 3.0 to 3.3 Mb in size whereas the smaller replicon varies considerably (0.8 to 2.4 Mb) amongst 34 species of vibrios, suggesting that this smaller chromosome has been more flexible in the evolution of *V. parahaemolyticus* (Okada *et al.*, 2005). Genome sequencing of a clinical strain, RIMD2210633, confirmed *V.
parahaemolyticus to contain two circular chromosomes and to possess 11 copies of ribosomal operons which correlate well with its rapid growth rates. Complete genome sequencing also revealed, for the first time, that *V. parahaemolyticus* harboured genes for type III secretion systems clustered together with pathogenicity islands and virulence effectors (Makino *et al.*, 2003). These are summarised in Table 2.1.

Table 2.1: Overview of *V. parahaemolyticus* genome organisation in a clinical strain RIMD2210633 (adapted from Makino *et al.*, 2003)

<table>
<thead>
<tr>
<th></th>
<th>Ch.1</th>
<th>Ch.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of sequence</td>
<td>3,288,558 bp</td>
<td>1,877,212 bp</td>
</tr>
<tr>
<td>G+C ratio</td>
<td>45.4%</td>
<td>45.4%</td>
</tr>
<tr>
<td>Open reading frames (ORFs)</td>
<td>3,080</td>
<td>1,752</td>
</tr>
<tr>
<td>Protein coding regions</td>
<td>86.9%</td>
<td>86.9%</td>
</tr>
<tr>
<td>Average ORFs length</td>
<td>926.9 bp</td>
<td>931.3 bp</td>
</tr>
<tr>
<td>rRNA (16S-23S-5S)</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>tRNA</td>
<td>112</td>
<td>14</td>
</tr>
<tr>
<td>Secretion systems</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Genomic Islands</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

2.1.3 Ecological niches

Coastal filter-feeding invertebrates such as oysters concentrate vibrios. However, the relationships between anthropogenic effects on coastal ecosystems (i.e. fish or shellfish farming), the natural hosts (i.e., filter feeders) and the environmental niches of vibrios are complex, dynamic and not very well understood. *V. parahaemolyticus* has been shown to possess multiple cell types that may explain its habitat in a variety of niches (McCarter, 1999). A planktonic free-swimming state, a sessile existence within a microbial community such as the commensal relationship in shellfish, or attached to under sea surfaces (boats, seaweeds, fishes etc) and attachment to host organism in pathogenesis are just some of the adaptations of this organism (Kaneko & Colwell, 1973, 1975). Extensive *in-vitro* studies have revealed two cell types; a "swimmer cell" and a "swarmer cell." (McCarter, 1999). The swimmer cell consists of a single polar flagellum, powered by the
sodium motive force (Atsumi et al., 1992) ideally suited for the survival and proliferation in the marine environment at a pH of approximately 8.0 (Kogure, 1998). The swarmer cell type is believed to be more adapted for growth and colonisation of V. parahaemolyticus on surfaces or viscous environments (McCarter, 1999). Electron micrographs show a differentiated cell type with numerous lateral peritrichously arranged flagella (Fig. 2.1).

![Electron micrograph of a swimmer cell (A) and swarmer cell (B) types of V. parahaemolyticus. Bar indicates approximately 1 mm. (Reproduced with permission; McCarter, 1999)](image)

Densities of V. parahaemolyticus in environmental and seafood samples vary considerably depending on water temperature, salinity, location, sample types, faecal pollution and the method used for analysis (Kelly & Stroh, 1988; DePaola et al., 1988). DePaola and colleagues showed a significant log_{10} correlation between seasonal abundance of V. parahaemolyticus, salinities and temperature in Alabama oysters (DePaola et al., 2003a). Total V. parahaemolyticus abundance was highest in May and June with sea temperatures over 25°C and salinities close to the optimum of 17 parts per thousand (ppt). It was estimated that an increase of one degree Celsius resulted in a 1.2 fold increase in the number of V. parahaemolyticus colony forming units (cfu) per gram of oyster homogenate. Similar findings in seasonal abundance have also been reported in several other studies (Kelly & Stroh, 1988; DePaola et al., 1988, 2000).
Despite a tropism for warmer temperatures and tropical climates, there are an increasing number of reports of pathogenic *V. parahaemolyticus* in colder climates such as Alaska and Southern Chile, that up until now were considered regions too cold to support the growth of this organism (McLaughlin *et al.*, 2005; Gonzalez-Escalona *et al.*, 2005a; Fuenzalida *et al.*, 2006) These recent findings suggest remarkable changes in the organism’s ability to quickly adapt, survive and proliferate in different niches. The prevalence of these pathogenic *V. parahaemolyticus* in colder climates may also be indications of global warming and thus an increasing cause for worldwide concern.

2.1.4 Viable but non-culturable state of *V. parahaemolyticus*

As with many human pathogens, *V. parahaemolyticus* is also thought to exist in the viable but non-culturable (VBNC) state and this may explain the low detection rates of *V. parahaemolyticus* in cold temperatures. For example, Baffone *et al.* (2006) recently identified unculturable *V. parahaemolyticus* in plankton fractions by PCR. Several laboratory studies have induced the VBNC state in *V. parahaemolyticus* by exposing cells to low temperatures under starvation conditions (Jiang & Chai, 1996; Mizunoe *et al.*, 2000; Wong & Wang, 2004). Temperature upshifts have been reported to “resuscitate” a sub-population of cells. In a recent study, VBNC *V. parahaemolyticus* that were subjected to temperature upshifts showed a remarkably heterogeneous population of cells that were coccoid or flatted in shape and in some cases showed asymmetrical cell divisions (Coutard *et al.*, 2007). These findings are also similar to VBNC in other *Vibrio* spp. (Vattakavan *et al.*, 2006; Pruzzo *et al.*, 2003). Remarkably, despite morphological and structural differences, the VBNC cells have been shown to retain their virulent properties (Bates & Oliver, 2004; Baffone *et al.*, 2003) thus the possible existence of environmental VBNC *V. parahaemolyticus* pose a significant public health concern and will hamper epidemiological investigations of this pathogen.
2.2 Worldwide distribution of *V. parahaemolyticus*

Food borne poisoning due to *V. parahaemolyticus* has been identified in many countries worldwide (Barker, 1974; Cabassi & Mori, 1976). Incidents are more common in countries when seawater temperatures rise over 15°C during the summer season and where seafood consumption is high (Alam *et al.*, 2003; DePaola *et al.*, 2003a; Lozano-Leon *et al.*, 2003).

2.2.1 Europe

In Europe, *V. parahaemolyticus* has been isolated in several countries including the UK (Hooper *et al.*, 1974; Ayres & Barrow, 1978), Demark (Kristensen, 1974), Norway (Gjerde & Boe, 1981; Bauer *et al.*, 2006) and Eastern European countries of Romania and Bulgaria (Aldova, 1989). However to date, most reports of the isolation of pathogenic *V. parahaemolyticus* are in southern Europe and the Mediterranean including France (Hervio-Heath *et al.*, 2002; Robert-Pillot *et al.*, 2004; Quilici *et al.*, 2005), Spain (Lozano-Leon *et al.*, 2003; Marne & Aznar, 1988; Martinez-Urtaza *et al.*, 2004, 2005; Molero, *et al.*, 1989), Italy and Greece (Masini, *et al.*, 2007).

In 1999 a large outbreak of *V. parahaemolyticus* related food poisoning occurred in Galicia, northwest Spain where a total of 64 people were admitted to hospital with acute diarrhoea after consumption of raw oysters (Lozano-Leon *et al.*, 2003). Temporary surveillance of *Vibrio* spp. employed at the time ensured the accurate identification of the etiological agent (Martinez-Urtaza *et al.*, 2004). Another outbreak involved 80 cases of illnesses associated with consumption of boiled crab (Martinez-Urtaza *et al.*, 2005). These sporadic cases in Spain may highlight possible deficiencies in the identification of *V. parahaemolyticus* by national reference laboratories and overall lack of surveillance and awareness of this pathogen.
2.2.2 East Asia

In Asia, where illness due to *V. parahaemolyticus* food poisoning is most prevalent, both pathogenic and non-pathogenic isolates have been characterised and reported in most countries including Korea, (Chun et al., 1974) Japan (Arakawa et al., 1999), Taiwan (Wong et al., 2005b), China (Yano et al., 2006) and Indonesia, (Marlina et al., 2007). For example, in Japan, where it was first recognised as a major cause of seafood related illness in 1950, an outbreak of 272 illnesses resulting in 20 deaths were associated with the consumption of raw sardines (Daniels et al., 2000). Since then, *V. parahaemolyticus* has been estimated to account for over 30% of all annual reported cases of food poisoning in Japan (Alam et al., 2002) and the leading cause of food poisoning (1710 incidents, 24,373 cases) between 1996 and 1998 as reported by the Infectious Disease Surveillance Centre (IDSC) of Japan (Su & Liu, 2007).

2.2.3 Southern Asia and Africa

Pathogenic isolates in the Indian subcontinent have been reported in an increasing number of environmental samples in Bangladesh (Islam et al., 2004), Sri Lanka (Palasuntheram et al., 1977), and India where, together with *V. cholerae* infection, fatalities have been the highest in the malnourished and immuno-compromised populations (De, 1977; Deepanjali et al., 2005; Parvathi, et al., 2006; Sen et al., 2007) (section 2.3). Gastroenteritis related to *V. parahaemolyticus* has also been reported in Africa, (Bockmuhl & Triemer, 1975) as well as an incident during the *V. cholerae* El Tor cholera epidemic in West Africa (Bockemuhl et al., 1975). A recent report also highlighted the presence of the more virulent pandemic strain (section 2.4.4) of *V. parahaemolyticus* in Mozambique. (Ansaruzzaman et al., 2005) (Fig. 2.2).
2.2.4 North America

Sporadic outbreaks of *V. parahaemolyticus* food poisoning have been reported in the USA since 1972 (Molenda et al., 1972). Between 1973 and 1998, 40 outbreaks of *V. parahaemolyticus* food poisoning were reported to the Centers for Disease Control (CDC) in the USA (Daniels et al., 2000). Among them, four major outbreaks involved over 700 cases of food poisoning due to raw oyster consumption, most prevalent during the warmer months of each year (Daniels et al., 2000; DePaola et al., 2000). Perhaps more significant for the North European community are the two recent reports of virulent *V. parahaemolyticus*, in Washington, British Columbia and Alaska (McLaughlin et al., 2005). Despite the colder Alaskan and Canadian sea water temperatures, *V. parahaemolyticus* related food poisoning resulted in 14 and 177 cases of infection, in 2004 and 2006 respectively (Khaira & Galanis, 2007; McLaughlin et al., 2005).

2.2.5 South America

Environmental reservoirs of *V. parahaemolyticus* were identified in South America as early as 1977 (Casellas et al., 1977; Franca et al., 1980). Several studies have indicated the virulence potential of *V. parahaemolyticus* (Magalhaes et al., 1992) with an outbreak of *V. parahaemolyticus* food poisoning amongst military personnel in Peru (Begue et al., 1995). More recently, Cordova and colleagues reported two outbreaks, in 1998 and 2004 in Chile (Cordova et al., 2002; Gonzalez-Escalona, et al., 2005a). Between November 1997 and March 1998, over 300 cases were reported in northern Chile with another outbreak affecting 1500 people between January and March 2004 in Puerto Montt, a region of usually cold coastal water (Gonzalez-Escalona et al., 2005a). A further outbreak between January and April 2005 in the same region resulted to 3,725 cases of acute diarrhoea. As Puerto Montt is one of the main shellfish producing areas of in Chile, contaminated shellfish had rapidly spread to other urban regions. By the end of March 2005, a total of 10,783 cases were reported, making it the largest documented occurrence of *V.
*parahaemolyticus* food poisoning in the world (Cabello *et al.*, 2007). The occurrence of these large outbreaks indicates that the contamination of *V. parahaemolyticus* in oysters is a great safety concern to the UK shellfish industry and public health.

### 2.3 Pandemic serotypes of *V. parahaemolyticus*

Antigenic serotypes based on the somatic (O) and capsular (K) antigen of *V. parahaemolyticus* have been described since 1969 (Miwatani *et al.*, 1969; Zen-Yoji *et al.*, 1970). To date, antiserum for 13 O and 71 K antigens have been reported and are commercially available to type *V. parahaemolyticus* isolates. Diverse serotypes have caused outbreaks worldwide. However, since 1996 an increasing number of cases are related to the O3:K6 serotype (Nair *et al.*, 2007). This serovar was first identified in Calcutta after a sudden increase in incidences during a hospital-based active surveillance program (Okuda *et al.*, 1997b). Between September 1996 and April 1997 this serovar accounted for 63% of all reported cases and since then this highly virulent serovar has dominated in a large number of outbreaks worldwide (Chiou *et al.*, 2000; Chowdhury *et al.*, 2000b). In the USA the largest outbreak of *V. parahaemolyticus* food poisoning, with 416 cases, was attributed to the O3:K6 serovar with the same serovar later involved in another outbreak in 1999 (Daniels *et al.*, 2000; Su & Liu, 2007). This serovar was also responsible for the outbreaks in Chile Gonzalez-Escalona *et al.*, 2005a), and Spain (Martinez-Urtaza *et al.*, 2005).

Since the year 2000, a number of reports have identified several other serovars prevalent in outbreaks. Amongst them, serovars of O1:KUT, O1:K25, and O4:K68, have been responsible for a large number of food poisoning cases worldwide (Chiou *et al.*, 2000; Chowdhury *et al.*, 2004b; Ansaruzzaman *et al.*, 2005; Rizvi *et al.*, 2006; Serichantalergs, *et al.*, 2007). All three serotypes have been shown to possess remarkable homology in molecular typing profiles to the O3:K6 serovar, thus it is believed that all three serovars of
O1:KUT, O1:K25, and O4:K68 are derived from the parent O3:K6, despite possessing different O and K antigens (Chowdhury et al., 2000a; Chowdhury et al., 2004a; Chowdhury et al., 2004b; Nair et al., 2007). Clearly, these changes in the O and K antigens demonstrate the dynamic nature of the evolution of virulent V. parahaemolyticus by the acquisition of O and K antigens.

2.4 Virulence factors and pathogenicity

2.4.1 Human infection

Clinical manifestations of V. parahaemolyticus infection follow an incubation period of between 12 – 46 hours and symptoms that include a self-limiting diarrhoea, abdominal cramps, nausea, vomiting, headache, fever and chills may last up to seven days (Nolan et al., 1984). Extra-intestinal infections have also been known to occur including wound, eye and ear infections (Tacket et al., 1982; Johnson, et al., 1984; Sautter et al., 1988; Kelly & Stroh, 1988). Between 1988 and 1997, 345 sporadic V. parahaemolyticus infections were reported to the CDC of which 45% were hospitalised (Daniels et al., 2000). Of these, 59% were gastroenteritis, 34% were wound infections, 5% were septicaemia, and 2% were from other exposures. In most cases of gastroenteritis, rehydration therapy is sufficient and antibiotic treatment is not required. For more serious infections tetracycline has long been the drug of choice, although several other antibiotics have commonly been used (Morris & Black, 1985). However, as in most cases of food poisoning, and due to the self-limiting symptoms of infection, mild cases of infections are not reported and consequently, actual figures of infection remain largely unknown.

Most strains isolated from infected patients demonstrate a major virulence factor known as thermostable direct haemolysin (TDH) that has been shown to exhibit β-haemolysis on Wagatsuma agar (Sakurai et al., 1973). This characteristic phenotype is also known as the Kanagawa phenomenon (section 2.4.2). Another virulence factor, TDH related haemolysin
(TRH), is typically associated with Kanagawa negative or urease positive isolates of *V. parahaemolyticus* (Eko, 1992; Kelly & Stroh, 1989; Suthienkul *et al*., 1995) and both TDH and TRH have been isolated from the same organism (Xu *et al*., 1994). However, most strains of *V. parahaemolyticus* isolated from the environment express neither TDH nor TRH even if they harbour the *tdh* or *trh* gene sequences respectively (Nishibuchi *et al*., 1989; Nishibuchi & Kaper, 1995). Furthermore, the infectious dose of pathogenic isolates may be high or low, with the more pathogenic strains of *V. parahaemolyticus* having been described as having multiple copies and varying expression of TDH and TRH (Honda *et al*., 1989a; Nishibuchi & Kaper, 1990; Nagayama *et al*., 1995).

### 2.4.2 Thermostable direct haemolysin (TDH)

The gene for TDH is present in 93-96% of clinical isolates and can be found in two or three copies either on the smaller chromosome flanking the pathogenicity island or on plasmids (Makino *et al*., 2003; Nishibuchi & Kaper, 1990; Baba *et al*., 1991a). The cloned *tdh* gene has a GC content of 35.6% and produces a 21.1 kDa homodimer protein product consisting of 189 amino acid residues including signal peptides (Taniguchi *et al*., 1985).

Although the mechanism of TDH action has not been fully elucidated, workers have described TDH as a pore forming toxin (Honda *et al*., 1992; Takahashi *et al*., 2006) forming a cation channel (Lang *et al*., 2004) and its most extensively studied effect is the ability to induce haemolysis of erythrocytes from different mammalian species (Huntley & Hall, 1994, 1996; Douet *et al*., 1996). Beta-haemolysis on specialised blood agar, Wagatsuma agar is the most common phenotype used to ascertain TDH positive isolates of *V. parahaemolyticus*. However it has been shown that expression of both *tdh* genes are required for an adequate β-haemolysis phenotype (Nishibuchi & Kaper, 1990). The workers showed that all cloned TDH genes exhibited at least 96.7% nucleotide sequence homology but *tdh* specific RNA transcripts were missing in some of the clones. Therefore,
differences at the transcriptional control level are likely to be responsible for the variation in haemolytic phenotypes. Clearly, the variability in haemolysis amongst different isolates needs to be addressed if the haemolytic activity is to be used as a virulence marker of V. parahaemolyticus (Chapter 4). Variants of TDH have also been isolated from V. cholerae non-O1, V. mimicus and V. hollisae (Nishibuchi et al., 1990; Baba et al., 1991a; Yoh et al., 1989; Terai, et al., 1990). The nucleotide sequences of various tdh genes are well conserved and their protein products immunologically indistinguishable (Nishibuchi & Kaper, 1995).

TDH has also been shown to induce cytotoxic effects consisting mainly of cell degeneration, which often leads to loss of viability (Sakurai et al., 1976). Using human and rat monolayers Raimondi et al demonstrated a TDH dose dependent increase in intracellular free Ca^{2+} and Cl' secretion leading to cell death (Raimondi et al., 2000). This work together with previous work reported by Tang et al, showed that cytotoxicity due to TDH is mediated by a Ca^{2+} independent pathway (Tang et al., 1995). The haemolysin TDH has also been described as a cardiotoxin found to stop the spontaneous beating of cultured rat and mouse myocardial cells (Goshima et al., 1976) and intravenous injection of TDH into rats resulted in rapid death through cardiac arrest (Honda et al., 1976).

2.4.3 Thermostable direct haemolysin related haemolysin (TRH)

Thermostable direct haemolysin related haemolysin (TRH) has typically been isolated from clinical cases of V. parahaemolyticus food poisoning in almost half of Kanagawa negative isolates (Yoh et al., 1992). More recently it has also been isolated from V. alginolyticus (Xie et al., 2005; Gonzalez-Escalona et al., 2006b). Other than a lack of β-haemolysis on Wagatsuma agar, TRH is similar in attributes to TDH and the proteins share up to 68% nucleotide sequence and 62% amino acid similarity (Xu et al., 1994; Nishibuchi et al., 1989). Up to two copies of variants trh1 and trh2 respectively have been reported in
a single genome (Kishishita et al., 1992) and in some cases is correlated with the presence of the urease gene cluster. Consequently, urea production may be used as a possible marker for TRH and potential virulence in *V. parahaemolyticus* (Eko, 1992; Kaysner et al., 1994; Suthienkul et al., 1995; Chiu et al., 2007). Iida et al have shown genetic linkage between the urease gene cluster, the genes *ure* and *trh* by identifying both *ure* and *trh* on the same *NotI* digested PFGE fragment (Iida et al., 1997; 1998). They were able to show that both *trh* and *ure* were within 8.5 kb of each other and that the *trh* and *tdh* genes were localized within a 40 kb region. A low and aberrant CG content relative to the genome in this region, and presence of phage-like integrases subsequently led to characterisation of several genomic islands (Hurley et al., 2006).

### 2.4.4 Other haemolysins in *V. parahaemolyticus*

Taniguchi et al described another thermostable haemolysin (delta-VPH) that was found in all clinical and environmental *V. parahaemolyticus* isolates (Taniguchi et al., 1990). This haemolysin had no homology to that of TDH or TRH and was also found present in *V. damselae* but its significance in human disease is not known. Similarly, Sakurai and colleagues demonstrated the presence of an additional haemolysin in *V. parahaemolyticus* that unlike TDH or TRH, was deactivated upon heating at 60 for 10 mins (Sakurai et al., 1974). The mature thermolabile haemolysin (TLH) was found to be of 45.5 kDa and its gene had a GC content similar to that of the genome (Taniguchi et al., 1986). Whilst TDH was found only in Kanagawa positive isolates, TLH was found in all strains tested (Sakurai et al., 1974). Since then, the gene for TLH has been used as a species specific marker for *V. parahaemolyticus* (section 2.6.3).

### 2.4.5 Cytotoxicity and enterotoxicity of *V. parahaemolyticus*

Both TDH and TRH, together with several uncharacterised factors have been shown to be responsible for the cytotoxic and enteropathogenic properties of *V. parahaemolyticus*. In
some early studies, Carruthers (1975) demonstrated cytotoxicity to HeLa cells and scanning electron microscopy of cells derived from human amniotic membrane revealed a decrease in microvilli and damage to cell morphology with sub lethal amounts of TDH. Higher dosage caused complete structural damage to the cytoplasm (Sakurai et al., 1976). Naim et al used osmotic stabilizers to show that the mode of action by TDH on erythrocytes was different from that on cultured embryonic fibroblast cells (Naim et al., 2001).

2.4.6 Other potential pathogenicity determinants

In one of the largest clinical studies to date, Suzuki and colleagues demonstrated that 3.7% of *V. parahaemolyticus* isolated from 1319 cases of traveller’s diarrhoea lacked both *tdh* and *trh* genes (Suzuki et al., 1997). Indeed, when *tdh* deletion mutants lacking both copies of *tdh* were created by homologus recombination, the mutants lost their haemolytic activity yet still demonstrated toxicity to HeLa cells as well as partial fluid accumulation in rabbit intestine (Park et al., 2004a). Therefore, it is likely that additional factors are involved in the pathogenicity of *V. parahaemolyticus*.

2.4.7 Serine protease

Work carried out by Lee et al. (2002) demonstrated a serine protease from a clinical strain lacking both *tdh* and *trh* genes. The protease showed significant effects on the growth of Chinese hamster ovary (CHO), HeLa, Vero and Caco-2 cells. The protease lysed erythrocytes, caused tissue haemorrhage and death in mice when injected both intraperitoneally and intravenously, but it was heat labile unlike TDH. It was shown that the serine protease was widely distributed in *V. parahaemolyticus* and *V. alginolyticus* (Ishihara et al., 2002).
2.4.8 Adherence and colonisation factors

An early report in 1977 demonstrated an increase in adherence by Kanagawa positive isolates to human foetal intestinal cells, whereas Kanagawa negative isolates adhered at a much slower rate or not at all on cultured HeLa cells (Carruthers, 1977). The work suggested a carbohydrate molecule on the surface of the Kanagawa positive bacterial cell wall was responsible for this interaction. However, no specific receptor molecules have yet been discovered although it has been reported that *V. parahaemolyticus* shows an increase in adherence under starvation conditions (Wong *et al*., 2005a). Nakasone *et al.* demonstrated that pili play a key role in the adhesion and colonisation in the rabbit intestine by isolating pili that were adhesive to the intestinal epithelium (Nakasone *et al*., 1990, 1992). These pili were found to be indistinguishable in morphology to pili of a pandemic O3:K6 strain, yet were antigenically distinct (Nakasone *et al*., 2000). Further work is required to establish mechanisms of *V. parahaemolyticus* adherence.

2.4.9 Siderophores

Several workers have described the potential role of siderophores in *V. parahaemolyticus* pathogenicity (Yamamoto *et al*., 1992b, 1994, 1995; Tanabe *et al*., 2003, 2006; Funahashi *et al*., 2003). In conditions of iron limitation, Dai *et al.* (1992) showed an increase in haemolytic activity of Kanagawa positive strains, together with the production of a siderophore, an increase in adherence, and lethality of *V. parahaemolyticus* to mice cells. Similar findings were obtained with Kanagawa negative isolates suggesting that virulence factors may be induced or enhanced under iron-limited conditions. The siderophore, named vibrioferrin (Yamamoto *et al*., 1994) was found to sequester iron from 30% iron saturated human transferrin for growth, suggesting that *V. parahaemolyticus* may be able to utilise host iron through the action of vibrioferrin for survival and proliferation (Yamamoto *et al*., 1994). Under iron limiting growth conditions, clinical isolates showed higher production of vibrioferrin than strains isolated from seafood thus suggesting a selective advantage of the.
survival of clinical isolates in the human intestine (Yamamoto et al., 1999). Although vibrioferin has been well characterised (Tanabe et al., 2003, 2006), and the receptor genes found widely distributed amongst *V. parahaemolyticus* (Funahashi et al., 2003) in both clinical and environmental isolates, its significance in the role of pathogenicity remains unclear.

### 2.4.10 Insertion Sequences

The possible role of insertion sequences in the spread of *tdh* genes was described by Terai et al. (1991) upon the characterisation of *tdh* in related species, *V. cholerae* non-O1, *V. mimicus* and *V. hollisae* (Nishibuchi et al., 1990). Analysis of 7 *tdh* genes and their flanking regions revealed insertion sequence like elements, termed insertion sequences of *Vibrio* species (ISVs) or sequences that were derived from the genetic rearrangement of the ISVs. The possible role of ISVs in transferring *V. parahaemolyticus* pathogenicity determinants have also been described by several others including (Burstyn et al., 1980; Baba et al., 1991a; Park et al., 2000) and Okura et al., (2005) who identified a large 16-kbp insertion sequence which was present in almost all pandemic strains but not in non pandemic strains tested.

### 2.4.11 Phage associated virulence

A large number of bacteriophages (vibriophages) with either a specific host or a broad host range have been described (Inoue et al., 1995; Hardies et al., 2003; Comeau et al., 2006), including those found in the pandemic O3:K6 clone (Nakasone et al., 1999; Chang et al., 1998). Indeed, Chang et al. (2000) showed that filamentous phage genomes of the previously described phages Vf12 and Vf33, integrated into genomic DNA of *V. parahaemolyticus* at distinctive regions and were also distributed on some plasmids and total cellular DNAs of one *V. damsela* and one non-agglutinable vibrio strain tested (Taniguchi et al., 1984; Chang et al., 1998). However, the potential of these phages as
tools for genetic transmission of virulence factors in *V. parahaemolyticus* has yet to be determined.

Within the pandemic O3:K6 serovars, the open reading frame ORF8, from filamentous phage f237, has been extensively characterised and has been described as a marker for pandemic strains (Nasu et al., 2000). The phage genome was originally found in a common plasmid pO3K6, in all 24 pandemic O3:K6 strains tested and genetic organisation resembled that of a previously characterised phage VF33 (Taniguchi et al., 1984). The double stranded DNA obtained from the treatment of f237 with DNA polymerase was identical to that of pO3K6 suggesting that pO3K6 is a replicative form of f237. The sequence of ORF8 (orf8) has since been used to identify pandemic strains by several workers (Myers et al., 2003; Laohaprertthisan et al., 2003) although there have been reports by others that ORF8 has poor correlation with the pandemic genotype (Bhuiyan et al., 2002; Okura et al., 2003). To date, there appears to be no evidence of lysogenic phage conversion, like that of the cholera toxin in *V. parahaemolyticus* (Waldor & Mekalanos, 1996)

### 2.4.12 Type III secretion systems

Type III secretion enables Gram-negative bacteria to secrete and inject pathogenicity proteins into the cytosol of host cells (Hueck, 1998). The presence of genes for a type III secretion system (TTSS) was first described upon genomic sequencing of *V. parahaemolyticus* (Makino et al., 2003) and since then TTSS genes have also been characterised in *V. cholerae* non-O1 and non-O139 (Dziejman et al., 2005). The TTSS is the principle virulence factor of diarrhoea causing bacteria such as *Shigella, Salmonella*, and enteropathogenic *Escherichia coli*, which cause gastroenteritis by invading or interacting with intestinal epithelial cells (Hueck, 1998). The presence of TTSS homologues was also demonstrated in other vibrios, such as *V. alginolyticus, V. harveyi*. 

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and *V. tubiashi*, suggesting that the TTSS may have a role in protein secretion in these organisms during interaction with eukaryotic cells (Park *et al.*, 2004b).

Park and colleagues characterised two sets of genes termed TTSS1 and TTSS2. By constructing mutant strains of TTSS1 and TTSS2 they were able to show reduced cytotoxic and enterotoxic activities compared with the parent strains (Park *et al.*, 2004b). Molecular characterisation of the TTSS1 of *V. parahaemolyticus* most closely resembled that of *Yersinia* spp., with over 30 gene homologues and a large inversion of the *yscA to yscQ* region together with an additional 12 hypothetical genes within the *ysc* region that are not found in *Yersinia* spp. or other species. Work carried out by Ono *et al.* (2006) later identified some of these genes to encode effector proteins found translocated into HeLa cells and that the cytotoxicity to HeLa cells was related to one of the newly identified secreted proteins encoded by VP1680 (Chapter 9). The second secretion system, TTSS2 was found on the smaller chromosome (section 2.1.2) in an area characterised as a pathogenicity island. In this region only 9 genes had homologues to *Yersinia* spp. and resembled the TTSS found recently on *V. cholerae* (Dziejman *et al.*, 2005).

Kodama *et al.* (2007) showed that TTSS2 induced a cytotoxic effect on human epithelial intestinal cell lines (HCT-8 and Caco-2) partly due to a putative ADP-ribosyltransferase. A further two TTSS2-dependent secretion proteins necessary for TTSS2-dependent cytotoxicity and enterotoxicity were also characterised (Kodama *et al.*, 2008). However, despite the functional characterisation of TTSS2, the distribution of TTSS systems remains unclear. According to Park *et al.*, (2004b) and Ono *et al.*, (2006) the TTSS2 was only found in Kanagawa positive clinical strains tested thus suggesting that TTSS2 had a major role in the pathogenicity of *V. parahaemolyticus* (Chapter 9).
2.5 Conventional methods for identification

2.5.1 Identification and detection methods of *V. parahaemolyticus*

*V. parahaemolyticus* is one of the fastest growing organisms with doubling times as short as 9 min in optimum growth conditions (Katoh, 1965). These include a pH of 7.8 to 8.6 and a temperature of 37°C, although it has been found to grow in a wide range of pH and temperatures (Yeung & Boor, 2004). This, together with a tolerance of moderately high alkaline conditions up to pH 9, forms the basis of a variety of selective media used to grow and enrich food samples and in use since 1971 (Twedt & Novelli, 1971).

2.5.2 Isolation

Isolation of *V. parahaemolyticus* from environmental and food sample usually requires homogenisation of sample with enrichment broths such as alkaline peptone water (APW) or glucose salt teepol broth (GSTB). Salt polymyxin broth (SPB) and salt colistin broth (SCB) containing the appropriate antibiotics for isolation of *V. parahaemolyticus* from other marine vibrios have also been used. Beuchat, (1977) identified other selective enrichment broths including 7%NaCl tryptic soy broth (TSBS), arabinose-ethyl violet broth (HAEB) and water-blue-alizarin yellow broth (WBAY) for the enrichment and recovery of cold- and heat-stressed *V. parahaemolyticus*. The use of a pre-enrichment step before selective enrichment has also been shown to increase *V. parahaemolyticus* counts (Ray *et al.*, 1978; Hofer & Silva, 1984; Hara-Kudo *et al.*, 2001).

2.5.3 Culture and growth

The most probable number method (MPN) described in the US Food and Drug Administration Bacterial Analytical Manual (Elliot *et al.*, 1995) is the most commonly used method for the detection of *V. parahaemolyticus*. The MPN method is both labour intensive and time-consuming (Su & Liu, 2007). Since 1963, thiosulphate citrate bile salt sucrose agar (TCBS) has been used widely as a selective medium for many *Vibrio* spp.
including *V. parahaemolyticus*. This medium uses a combination of ox bile, NaCl, a high pH and an indicator system for acid production from sucrose. However, TCBS cannot differentiate *V. parahaemolyticus* from other *Vibrio* species that may be sucrose negative such as *V. mimicus* or *V. vulnificus*. Several workers have evaluated TCBS supplied by different manufacturers to reveal inconsistencies in the recovery and quality of growth of each species (West *et al.*, 1982) suggesting the need for better quality controls. Due to the common presence of more than one *Vibrio* species in environmental and food samples, it may be more favourable to use TCBS in clinical cases than culturing from environmental samples.

*V. parahaemolyticus* has also been isolated on a variety of other media including mannitol salt agar (Carruthers, 1976), sucrose teepol tellurite agar (Chatterjee *et al.*, 1977), and thiosulphate chloride iodide agar (Pfeffer & Oliver, 2003) although none have been used extensively. Tryptic soy agar containing triphenyl tetrazolium was developed to enhance differentiation between *V. parahaemolyticus* and *V. alginolyticus* (Kourany, 1983) but also does not appear to have been widely used. The performance of CHROMagar Vibrio (CV), a medium containing a chromogenic beta-galactosidase system was also studied (Hara-Kudo *et al.*, 2001). In comparison to TCBS, a greater proportion of samples were positive in CV and less interference from other vibrios as *V. parahaemolyticus* appears violet in colour whilst other vibrios appear pale blue. Unlike TCBS, the colour of the colonies did not change with prolonged incubation or storage.

### 2.5.4 Biochemical profiling

The screening of presumptive *V. parahaemolyticus* is necessary to exclude as many non-target organisms as possible before further identification. These basic screening tests include assessing cell growth, morphology, motility, swarming on growth surface, Gram staining and several biochemical tests including arginine dihydrolase (ADH) and Voges-
Proskauer tests. Kaper et al. (1979) described a medium for screening of presumptive *V. parahaemolyticus*. This includes seven tests in one medium including the utilisation of mannitol, sucrose and lactose together with ADH, indole reactions, gas and H₂S production. Although it was able to differentiate *V. parahaemolyticus* from other *Vibrio* spp. some combinations of positive reactions were found to interfere with detection of other tests.

A full list of biochemical tests differentiating between closely related species have been described (Elliot et al., 1995). Of the many tests it has been noted that an increasing number of *V. parahaemolyticus* are resistant to 150μg of di-isopropyl pteridine phosphate (O/129). Several workers have also reported oxidase negative and urease positive strains that coincide with pathogenic *V. parahaemolyticus* (Eko, 1992).

### 2.5.5 API and commercial identification systems

The use of API 20E and API 20NE (BioMerieux) has been shown to be the most reliable and quickest method to identify *V. parahaemolyticus* (Overman et al., 1985, 1986). In the most recent study, Croci et al., (2007) evaluated API 20E, API 20NE and a set of 32 conventional tests described previously by Alsina and Blanch (1994) for the rapid identification of vibrios. Intra- and inter-laboratory test findings showed the Alsina and Blanch scheme to be the most sensitive in correctly identifying *V. parahaemolyticus*. Veretta et al. compared the API20E and API20NE identification systems to a BBL Crystal enteric/nonfermenter (E/NF) identification system using Gram negative rods from clinical samples (Varettas et al., 1995). The workers found that the Crystal system was more convenient than API for routine clinical use because it required fewer repeats and was easier and less time consuming to use. O’Hara and co-workers applied these and several other systems that included MicroScan Neg ID2, Rapid Neg ID3, Vitek GNI+ and ID-GNB to identify members of the *Vibrionaceae* family (O’Hara et al., 2003) with varying
levels of accuracy. Overall percentage accuracy ranged from 63.1% to 80.9% with the MicroScan Neg ID2 and Crystal E/NF respectively.

In what appears to be the first study of its kind on *V. parahaemolyticus*, Martinez-Urtaza et al. (2006) used an API 20E system to biochemically characterise differences between clinical and environmental isolates of *V. parahaemolyticus*. By using 2% and 0.85% NaCl as diluent (as recommended by the manufacturer), they showed that environmental isolates gave more accurate identification using a 2% NaCl, whereas clinical isolates were correctly identified using 0.85% NaCl, consistent with previous findings (MacDonell et al., 1982). Additionally, clinical and environmental isolates demonstrated significant differences in up to 5 tests. Both gelatine utilisation (GEL) and arabinose fermentation (ARA) were positive in the environmental group and negative in the clinical isolates tested. Environmental isolates analysed using 2% NaCl showed higher positive reactions for citrate utilisation, urease, melibiose and amygdalin than those using 0.85% NaCl thus suggesting small yet significant differences in metabolism amongst clinical and environmental isolates.

### 2.5.6 Characterisation of pathogenic *V. parahaemolyticus*

The difficulty in obtaining reliable identification of *V. parahaemolyticus* by conventional biochemical procedures alone is widely recognised and summarised above. Once identified, there is no biochemical test or series of tests that would confirm a pathogenic isolate. However, the haemolytic properties of TDH have been well documented and correlated with human pathogenicity since 1969 (Miyamoto et al., 1969) and β-haemolysis of human or rabbit erythrocytes on Wagatsuma agar is a commonly used indicator for TDH related virulence (section 2.4.2). The related haemolysin, TRH, is more active against calf and sheep red blood cells than human or rabbit cells but does not produce haemolysis on
Wagatsuma agar although the presence of TRH has been associated with the production of urease in some findings (section 2.4.3, Eko, 1992; Iida et al., 1997; Park et al., 2000).

2.5.7 Immunological detection of pathogenic \textit{V. parahaemolyticus}

Honda \textit{et al.} modified a BTB-Teepol agar and arabinose-ammonium sulphate-cholate agar (MAAC) for the immunological detection of TDH producing isolates (Honda \textit{et al.}, 1980, 1982). Using a modified Elek test and immunohalo test with antiserum or anti haemolysin IgG, it was possible to identify the Kanagawa phenomenon on a single plate. Workers of the same group later developed several ELISA methods for the detection of TDH and TRH (Honda \textit{et al.}, 1985, 1989b, 1990) and subsequently developed and evaluated two assay kits for TDH and TRH that are commercially available (Yoh \textit{et al.}, 1995). The two kits, reversed passive latex agglutination (RPLA) and Bead-ELISA, although originally developed for detection of TDH, were found to detect TRH with a minimum concentration of 10 ng/ml.

2.6 Molecular detection methods

2.6.1 PCR methods for \textit{V. parahaemolyticus}

A number of successful PCR-based assays have been developed to assist in the specific detection of \textit{V. parahaemolyticus} and its toxin genes. Most of the species specific gene targets used by workers include the genes \textit{toxR}, \textit{tlh}, and \textit{gyrB}, the genes encoding for ToxR transmembrane protein, thermolabile haemolysin (section 2.4.4) and DNA gyrase subunit B respectively (Kim \textit{et al.}, 1999; Davis \textit{et al.}, 2004; Venkateswaran \textit{et al.}, 1998). Other gene targets that have been used with varying degrees of success include the heat shock protein \textit{hsp60} gene, the species specific \textit{pR72H} fragment and to some extent the 16S- 23S spacer regions (Kwok \textit{et al.}, 2002; Lee \textit{et al.}, 1995; Sparagano \textit{et al.}, 2002). Several of these gene targets have been used collectively as multiplex PCR systems and quantitative real-time PCR (QRT-PCR) methods.
2.6.2 toxR gene based PCR methods

ToxR is a trans-membrane protein and the transcriptional activator of Vibrionaceae (Miller et al. 1987; Lin et al., 1993). It has been found in many Vibrio spp. and shown to be well conserved amongst its members (Higgins et al., 1996; Li et al., 2000; Okuda et al., 2001). However, some variations in sequence identity and divergence of toxR (Fig. 2.4) allow the design of PCR primers for specific Vibrio spp (Osorio & Klose, 2000).

![Figure 2.3: Unrooted trees constructed by the neighbor-joining method showing the phylogenetic interrelationships of the different Vibrio and Photobacterium species based on toxR gene nucleotide sequences (A) and 16S rRNA genes (B) (Osorio & Klose, 2000)](image)

Using PCR targeted to toxR, Kim et al. (1999) showed that all 373 V. parahaemolyticus isolates tested were positive for toxR and that the gene was present in other members of Vibrio spp. Some of the PCR reactions exhibited amplicons of different sizes and intensities suggesting toxR sequence variation in Vibrio species that may reflect phylogenetic relationships (Fig. 2.3). This was shown to be true in a follow up study by Osorio & Klose (2000). The toxR region has been widely used as a species specific marker in various PCR protocols including multiplex PCRs and real time PCRs (Kim et al., 1999; Lin et al., 1993; Alam et al., 2002; Dileep et al., 2003). In a comparison with toxR and two other loci (thl and pR72H) Croci et al. were able to show that toxR provided the best
results in the identification of *V. parahaemolyticus* with an inclusivity of 96%, no false positives, and a concordance between repeated determinations of 97% (Croci *et al.*, 2007).

### 2.6.3 PCR targeted to *tlh*

Several workers have used *tlh*, the gene for thermolabile haemolysin (section 2.4.4) as a species specific marker (Bej *et al.*, 1999; Kaufman *et al.*, 2002, Miwa *et al.*, 2003). Work by McCarthy *et al.* demonstrated the use of an alkaline phosphase and digoxygenin (DIG) labelled *tlh* probe that worked equally as well as the *tlh* PCR protocol (McCarthy *et al.*, 1999). The *tlh* PCR based probe showed 97% agreement with the API 20E diagnostic strips in the identification of *V. parahaemolyticus* (section 2.5.5) and was found to be considerably more efficient and reduce time and effort. In addition to the *tlh* PCR, several other targets such as a collagenase and metalloprotease gene have also been used as targets for the identification of *V. parahaemolyticus* with varying degrees of success (Di Pinto *et al.*, 2005; Luan *et al.*, 2007). However, these targets have yet to be assessed and validated by others.

### 2.6.4 Primers for *gryB*

Venkateswaran and workers developed *V. parahaemolyticus* specific DNA gyrase subunit B (*gryB*) primers. They showed that *V. parahaemolyticus* and *V. alginolyticus* shared a 86.8% homology in nucleotide sequences and developed a set of primers that correctly detected 117 strains of the target strain from 267 non-target strains (Venkateswaran *et al.*, 1998). Using sequence data from *gryB*, and other genes from multilocus sequence typing (section 2.7.10) Chowdhury *et al.* were able to assess evolution of the pandemic O3:K6 strains that had arisen since 1996 (Chowdhury *et al.*, 2004b). Other workers have developed multiplex, microarray and real-time PCR assays for the specific detection of *V. parahaemolyticus* (Gonzalez *et al.*, 2004; Cai *et al.*, 2006).
2.6.5 PCR of pR72H

Several workers have used the pR72H fragment obtained from a HindIII DNA digest of *V. parahaemolyticus* DNA cloned into pUC119. This fragment was found in all strains of *V. parahaemolyticus* tested and thus it was recommended as a species specific DNA probe. (Lee *et al.*, 1995; Croci *et al.*, 2002, 2007) Using this fragment Hervio-Heath and colleagues identified a novel 320bp amplicon, atypical of the 387bp fragment usually found, in 24.4% of the *V. parahaemolyticus* isolates (Hervio-Heath *et al.*, 2002). Workers in Chile identified two other amplicons using the same primers; a 400bp fragment and a 340bp fragment in both clinical and environmental samples. However, a third group of isolates of *V. parahaemolyticus* did not show a PCR band (Cordova *et al.*, 2002) and consequently the use of pR72H as a species specific marker remains questionable.

2.6.6 PCR methods for toxin genes

PCR for *tdh* and *trh* have widely been used both as a marker for pathogenic isolates in environmental samples as well as confirmation in clinical studies (Lee *et al.*, 1993; Karunasagar *et al.*, 1996; Wong *et al.*, 1999a; Alam *et al.*, 2002; Robert-Pillot *et al.*, 2004). The most widely used sets of primers are those developed by Tada *et al.* (1992) that target the *tdh2* and *trh1* regions. The gene *tdh2* is the more representative of *tdh* genes and is found to be more strongly associated with haemolysis of Kanagawa positive isolates than *tdh1* genes (Nishibuchi & Kaper, 1990; Iida *et al.*, 1990). Although the haemolysin TDH can be assayed using immunological tests and to some extent Wagatsuma agar, to date there appears to be no conventional tests for TRH production.

2.6.7 Multiplex PCR

The development of multiplex PCR (M-PCR) for pathogenic isolates have been reported by several workers using both *tdh*, *trh* and several species specific genes (Bej *et al.*, 1999; Kong *et al.*, 2002; DePaola *et al.*, 2003b; Okura *et al.*, 2003; Panicker *et al.*, 2004; Tarr et
Using M-PCR Lee et al. (2003) showed the detection of *V. parahaemolyticus* and related species from shellfish using *tl* (*tlh*) primers. The amplicons were then subjected to colorimetric CovaLink™ NH microwell plate sandwich hybridisation using phosphorylated and biotinylated *tlh* probes.

Panicker et al. (2004), used *tdh, trh, tlh* and *orf8* amplicons derived from a multiplex PCR as targets for oligonucleotide probes. The probes were deposited onto Teflon-masked slides by using a MicroGrid II arrayer and hybridisation detected by using tyramide signal amplification with Alexa Fluor 546 fluorescent dye. This microarray approach detected $10^2$ to $10^3$ CFU/ml of target organism in pure cultures and 1 CFU in 1 g of oyster tissue homogenate after 5h of sample enrichment with 100% specificity. Consequently, the authors suggested the use of a microarray approach together with multiplex PCR for efficient detection of pathogenic *V. parahaemolyticus* from shellfish samples.

**2.6.8 DNA hybridisation probes and ELISA methods**

DNA-DNA hybridisation probes have been used extensively as they can usually be applied directly onto plate grown cultures allowing identification of target genes and test all the colonies on a plate at the same time. Both digoxigenin (DIG) and alkaline phosphatase-conjugated probes have been used by several workers to target species specific and virulence genes (Yamamoto et al., 1992a; McCarthy et al., 1999; Gooch et al., 2001; Nordstrom et al., 2006; Raghunath et al., 2007). In comparison with API 20E identification strips, McCarthy et al. showed that specificity in identifying *V. parahaemolyticus* using the alkaline phosphatase probe and DIG-labelled method was 97% and 98% respectively. However, although the probes were specific, the sensitivity of this detection method was less than expected (McCarthy et al., 2000; Gooch et al., 2001). Lee et al. subsequently developed a CovaLink™ NH microwell plate approach using *tlh* primers that was reported to detect $10^2$ cells/g of oyster tissue homogenate (Lee et al., 2003) and offered a shorter
hybridisation time, higher sensitivity and high throughput sample processing, although this
has not been tested with pathogenic isolates or using \textit{tdh} or \textit{trh} probes.

\subsection*{2.6.9 Quantitative real-time PCR (QRT-PCR)}

Increasingly, a number of workers have reported QRT-PCR methods using various
markers including \textit{tdh}, \textit{trh}, \textit{tlh}, \textit{toxR}, \textit{gyrB} and both fluorescent and TaqMan probes (Davis
\textit{et al.}, 2004; Iijima \textit{et al.}, 2004; Kaufman \textit{et al.}, 2004; Takahashi \textit{et al.}, 2005; Cai \textit{et al.},
samples that were enriched in alkaline peptone water (APW). In comparison to using a
streak plate method followed by \textit{tdh} probe, the 24 hour QRT-PCR method was able to
detect the gene \textit{tdh} in 61 samples, whereas previously the streak plate/probe method
detected \textit{tdh} in 15 samples, a procedure reported to take 3 days (Blackstone \textit{et al.}, 2003).
Similarly, Bej and coworkers multiplexed four targets \textit{tdh}, \textit{trh}, \textit{tlh} and \textit{orf8}, for a count of
total and potentially pathogenic \textit{V. parahaemolyticus} for a QRT-PCR procedure that was
sensitive for 200 pg of purified genomic DNA and $10^4$ CFU per ml for pure cultures (Ward
\textit{et al.}, 2006; Rizvi \textit{et al.}, 2006). The high sensitivity of QRT-PCR was also demonstrated
by Nordstrom \textit{et al.} (2007) to detect total and potentially pathogenic \textit{V. parahaemolyticus}.

\subsection*{2.7 Strain differentiation by molecular typing methods}

\subsection*{2.7.1 Epidemiological typing of clinically significant strains}

Due to the large number of outbreaks of \textit{V. parahaemolyticus} food poisoning worldwide
and an increasing number of environmentally isolated, potentially pathogenic strains, there
have recently been numerous strategies developed to delineate patterns of transmission. In
some cases, typing has contributed to epidemiological surveillance and evaluation of
control measures by assessing prevalence and distribution of epidemic and pandemic
clones. Several molecular typing techniques for \textit{V. parahaemolyticus} are under
development and more recently, there has been a growing concern for standardised typing methods for *V. parahaemolyticus*.

**2.7.2 Principles and properties of a molecular typing method**

The accurate recognition and differentiation of subtypes within a bacterial species and the use of subtype identification methods to determine sources of infection, rates of spread of infection and mechanisms of transmission is referred to as typing. The basis for all molecular typing methods is that an outbreak of an infectious disease is the result of exposure to a pathogen that is derived from a single cell or clone and its subsequent progeny will be genetically identical or closely related to the source organisms (Streulens, 1998). The assumption is that at species level there is sufficient genetic diversity so that organisms isolated from different sources and/or at different times and locations can be classified as different isolates, subtypes or strains (Olive & Bean, 1999). The criteria for evaluating performance of typing systems may include assessments on typeability, discriminatory power, reproducibility, stability, epidemiological concordance and comparative study (Streulens, 1998).

Ideally, the techniques used in a molecular typing system should be standardised and reproducible so that they can be carried out in any laboratory and give informative and comparable data, which is applicable to all isolates. The system should be sufficiently discriminatory to distinguish a reasonable number of types amongst which isolates can be roughly equally distributed. Furthermore, a typing system should be able to differentiate unrelated strains such as those which are geographically distinct from the source organisms but at the same time demonstrate the relationships that exist between organisms isolated from individuals infected through the same source (Olive & Bean, 1999).
2.7.3 Pulsed field gel electrophoresis (PFGE)

Over recent years, pulsed field gel electrophoresis (PFGE) has emerged as the "gold standard" in molecular typing methodology and is the current method of choice for typing nosocomial and community acquired pathogens (Tenover et al., 1997). PFGE is an extremely sensitive and sophisticated method that uses whole intact genomic DNA prepared by embedding bacteria in agarose plugs. Bacterial cells are lysed in situ, followed by restriction enzyme digestion of the intact genomic DNA with an infrequently cutting enzyme after removal of proteins. The large DNA fragments generated are then separated in highly purified agarose using specialist gel electrophoresis systems. Differences in the length and number of restriction fragments arise as a result of the changes in restriction enzyme sites caused by duplication, mutation, deletion, insertion or inversion (Tenover et al., 1997).

PFGE has been used widely in regions of South East Asia where gastroenteritis due to *V. parahaemolyticus* food poisoning is endemic. Selections of different restriction enzymes including *NotI*, *SfiI* and *ApaI* have been used with various protocols and pulse times (Bag et al., 1999; Wong et al., 1999a; Lu et al., 2000; Martinez-Urtaza et al., 2004; Yeung et al., 2002; Chowdhury et al., 2004a; Tanil et al., 2005). One of the first reports of an extensive use of PFGE for subtyping of *V. parahaemolyticus* was reported for 130 clinical isolates in Taiwan in 1996 (Wong et al., 1996). Although comprehensive fingerprinting data were not presented, they reported clustering of the 130 isolates into 14 PFGE types one of which seemed to be clonally related to an isolate from Japan. Conversely, clonal diversity within the serotype O3:K6 was demonstrated by Bag and colleagues (1999), who combined the use of PFGE with ribotyping (section 2.7.4). Although there had been genetic reassortment among the 30 clinical O3:K6 strains tested, they were still predominantly one clone. A similar study incorporated the use of an arbitrarily primed PCR (AP-PCR) method with PFGE to identify a second serovar, O4:K68 in Thailand and
India that shared 78-91% genomic similarity to that of O3:K6 strains (Chowdhury et al., 2000a). Using PFGE, Okura et al. (2003) also suggested that the O1:K25, O1:K26, and O1:K untypeable pandemic serovars were derivatives of one clone.

Since the use of PFGE in typing *V. parahaemolyticus* in 1996, a number of methods with variations in the use of restriction enzymes (*Not*I, *Sfi*I and *Apa*I), pulse switch times, equipment and other parameters have been employed with varying degrees of success (Marshall et al., 1999). The main concern is a lack of typeability as DNA degradation occurs over the long preparation and processing times, not just in *V. parahaemolyticus* but also in *V. cholerae* and *V. vulnificus* (Gonzalez-Escalona et al., 2007). A number of workers have recently adopted the ‘One day (24-28 h) Standardised Laboratory Protocol for Molecular Subtyping of Non-typhoidal Salmonella by PFGE’ (PulseNet; CDC, Atlanta, Ga) with increased typeability. However, with an increasing number of outbreaks worldwide, and with PFGE as the preferred method of subtyping, there have been growing concerns for a standardised PFGE *V. parahaemolyticus* protocol to facilitate inter-laboratory strain comparisons. To this end, two standardised PFGE procedure for *V. parahaemolyticus* have recently been described (Parsons et al., 2007; Wong et al., 2007) and remain to be validated by others.

2.7.4 Restriction fragment length polymorphism (RFLP) analysis

Restriction fragment length polymorphism (RFLP) analysis has been used on a variety of occasions, usually in conjunction with other methods (Gendel et al., 2001; Yeung et al., 2002; Wong et al., 1999c). RFLP analysis uses restriction enzymes to digest genomic DNA and the resulting fragments are separated according to size by agarose gel electrophoresis in much the same way as PFGE without the need for sophisticated gel electrophoresis apparatus. This method has been used to characterise polymorphisms in virulence genes *tdh* and *trh* (Suthienkul et al., 1996) and genes encoding for components of
polar flagellar, Fla locus of *V. parahaemolyticus* (Marshall *et al.*, 1999). However, because of the complexity of the resulting pattern due to a large number of restriction fragments in a genome, the DNA is transferred to a membrane (i.e. Southern blot) and probed by DNA hybridisation using a labelled probe. Several probes have been used to characterise *V. parahaemolyticus*, the most common being ribosomal based probes (ribotyping), (Wong *et al.*, 1999b).

In what appears to be the first of two comparative studies of several molecular typing techniques, Marshall and colleagues used the *rrnB* rRNA operon of *E. coli* to ribotype 60 isolates (Marshall *et al.*, 1999). Although full genotypic data was not shown, *BglII* digested genomic DNA was reported to reveal 14 different types in comparison to 15 and 13 types as shown by PFGE and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR, section 2.7.8) respectively. Using Simpson’s index of diversity (DI) described by Hunter and Gaston (1988), the authors concluded that both ribotyping and ERIC PCR (section 2.7.8) provided the best discrimination, similar to that of PFGE discrimination while Fla locus RFLP analysis provided the lowest discrimination index (Marshall *et al.*, 1999). Another method employing RFLP that does not appear to have been assessed in *V. parahaemolyticus* include the use of insertion sequence (IS) probes, especially as there is evidence to suggest that *tdh* has been found to be spread by insertion sequence like elements (ISVs) (Baba *et al.*, 1991b; Terai *et al.*, 1991). IS probes such as IS200 have been used successfully to type *Salmonella* spp, *Streptococcus pneumoniae* (IS IS1167) and *Mycobacterium tuberculosis* (IS6110) (Stanley & Saunders, 1996; Luna *et al.*, 2000).

### 2.7.5 Randomly amplified polymorphic DNA (RAPD) PCR

Randomly amplified polymorphic DNA PCR (RAPD) is a widely used method that examines nucleotide variation throughout the entire genome and can be more discriminatory than methods that examine variation at a specific locus, particularly in
In one of the first reported studies of RAPD-PCR with *V. parahaemolyticus*, Wong *et al.* screened 100 different 10-mer primers (UBC RAPD primer set 100/3, Finnzymes Oy, Espoo, Finland) with a single clinical and environmental strain and proceeded to type 308 clinical isolates to identify 41 polymorphic RAPD patterns (Wong *et al.*, 1999). Although quality of data was not apparent, the RAPD clustering identified 5 major clinical subtypes that accounted for 91% of all the isolates and were phylogenetically more closely related to each other indicating a largely homogeneous group amongst the clinical strains tested. In another study that involved 1500 environmental isolates of *V. parahaemolyticus* in Bangladesh, the workers identified and characterised five *tdh* positive and clinically significant strains (Islam *et al.*, 2004). These five isolates were found to belong to three different serotypes; two that belonged to O3:K6 however, believed to be clonally related, showed identical RAPD bands. Similar results were also published by Hayat *et al.* (2006) who showed 0.3% of environmental isolates to be toxigenic and to possess identical RAPD bands to that of pandemic reference strains. Similar findings were reported by others on the application of RAPD using arbitrary primers to distinguish between *V. parahaemolyticus* strains (Matsumoto *et al*. 2000; Chowdhury *et al*., 2000b; Okura *et al*., 2003). These methods employed one or more of the primers provided with the Ready-To-Go RAPD Analysis Kit (Amersham Biosciences). However, a low number of amplicons and the use of different control strains do not facilitate meaningful comparison of data.
2.7.6 Ribosomal DNA intergenic spacer (ITS) region analysis

The genes encoding subunits of bacterial rRNA are typically arranged in a characteristic formation, 16S, 23S and 5S. Regions between the 16S and 23S rRNA genes and between 23S and 5S rRNA genes are known to be more variable in both size and nucleotide sequence than the flanking genes and are termed ITS1 and ITS2 respectively. The acronyms, IGS (intergenic spacer) and ISR (intergenic spacer region) have also been frequently used. Within a species, the 16S rRNA is often highly conserved with variable regions often used for phylogenetic and taxonomic purposes. The intergenic spacer region shows greater variability and can be used to distinguish between closely related bacterial species and, sometimes, between strains and isolates. A number of copies of the rRNA operon are known to exist throughout the genome in different species and this may provide adequate variation for epidemiological typing purposes (Gurtler & Stanisich, 1996).

Variation in the ITS1 and ITS2 regions have been the basis for the development of a number of subtyping systems for a wide number of species although sequence variation in the ITS1 region has been more widely used due to its larger size and consequently higher probability of nucleotide polymorphisms. In the recent genome sequencing project of *V. parahaemolyticus*, 11 rRNA operons (rrn) that fell into 6 classes were identified (Makino *et al.*, 2003) with identical 16S rRNA genes (rrs) (Gonzalez-Escalona *et al.*, 2005b). Although this large number of operons may produce complex ITS1 banding patterns, the relative frequency and distribution of rRNA genes throughout the genome amongst strains of *V. parahaemolyticus* may provide a significant tool for intraspecies subtyping of this organism. For example, a strain of *V. parahaemolyticus* that had been isolated in 1951 only contained two rrn sequence types, one of which was found in seven of the 11 rrn operons and the other in four (Maeda *et al.*, 2000).
The ITS1 region was first analysed in *V. cholerae* by Coelho and colleagues (1995) who used primers that flanked 16S and 23S rDNAs of *E. coli* to show that ITS1 PCR patterns from O1 classical, O1 El Tor and 0139 strains were different and therefore provide a potential tool for studying the epidemiology of *V. cholerae*. In a follow up study by Chun *et al.*, (1999), *V. cholerae* and *V. mimicus* ITS1 regions were studied using more specific primers based on sequences from *E. coli*, *Pseudomonas aeruginosa*, *V. cholerae* and *V. vulnificus* (Chun *et al.*, 1999). Perhaps not surprisingly due to a high specificity of primers and the use of only 10 isolates in the study, only four ITS1 variants were identified in both *V. cholerae* and *V. mimicus* where up to 10 *rrn* operons had previously been described (Nandi *et al.*, 1997).

2.7.7 Transfer DNA-PCR analysis

Conserved genes encoding tRNAs can be found widely distributed throughout a genome, usually clustered in multiple tandem-repeat units (Green & Vold, 1993). DNA that encodes for RNA is termed tDNA and the intergenic space between these tRNA genes can vary considerably between species and sub species (Welsh & McCelland, 1991a). tDNA gene regions appear to be hot-spots for the integration of prokaryotic genetic elements (Reiter *et al.*, 1989; section 2.4.10, insertion sequences) and has been shown in *E. coli* to confer specific traits such as enhanced virulence, antibiotic resistance and fitness traits necessary to adapt to certain niches (Dobrindt *et al.*, 2004; Hayashi *et al.*, 2001; Welch *et al* 2002). Low stringency PCR products based on primed tDNA regions can amplify comparable inter-length polymorphisms (tDNA-ILPs) between different strains and species and can be used to assess genome wide variation (Welsh & McCelland, 1991a). tDNA-ILPs have been used by several workers to rapidly subtype a wide variety of bacterial species including *Acinetobacter* spp., *Staphylococcus* spp., *Renibacterium salmoninarum*, *Enterobacter cloacae* and *Lactobacillus* spp., (Ehrenstein *et al.*, 1996, Maes *et al.*, 1997; Alexander *et al.*, 2001; Clementino *et al.*, 2001, Baele *et al.*, 2002).
Welsh and McClelland developed a set of 'consensus' tRNA gene primers from multiple alignments of tRNA genes from a wide variety of bacterial species including *E. coli*. The primers were designed to face outwards from the end of the tRNA genes to amplify a tDNA-ILP fingerprint determined by the arrangement of tRNA genes on the bacterial genome. Due to the conserved order and arrangement of tRNA genes on the genome, the banding profiles that are generated using consensus primers are often characteristic of a particular species and can be identical or nearly identical within every species (Welsh & McCelland, 1991a, 1992). Considering the relatively large number of tRNA genes that have been found in the *Vibrionaeae* (Maeda *et al.*, 2000), and in particular, 126 in *V. parahaemolyticus* (Makino *et al.*, 2003) that coincide with multiple *rrn* operons, tDNA-ILPs show significant potential as a method of subtyping *V. parahaemolyticus*.

### 2.7.8 Repetitive extragenic palindromic sequence PCR (REP-PCR)

Repetitive extragenic palindromic (REP) sequences are repetitive elements that also appear to be distributed throughout bacterial genomes and consist of a conserved 33 bp inverted repeat region (Stern *et al.*, 1984). The function of the REP sequence in the prokaryotic genome remains largely unknown although the sequences appear to stabilise upstream mRNA regions and influence gene expression (Newbury *et al.*, 1987). Although a high discrimination index was shown by Wong *et al.* (2001) in subtyping *V. parahaemolyticus*, Maluping *et al.* demonstrated less reproducibility even though the same primers and PCR conditions were used (Maluping *et al.*, 2005).

Another system that employs the use of conserved regions that have widely been used for bacterial typing includes ERIC-PCR. These sequences are a family of repetitive elements that were first described in the genomes of *E. coli* and *Salmonella Typhimurium* and also found in other species including *V. cholerae* (Hulton *et al.*, 1991). The sequence is 126 bp long and has been shown to be highly conserved and restricted to transcribed regions of the
genome, either in the intergenic regions of polycistronic operons or in the untranslated regions that lie upstream or downstream of open reading frames (Debruijn, et al., 1992). Although ERIC sequences are highly conserved, their chromosomal locations are known to vary between different bacterial species. ERIC-PCR has been widely used to characterise V. cholerae since 1991 and more recently used for V. parahaemolyticus with varying degrees of success (Khan et al., 2002; Maluping et al., 2005; Tanil et al., 2005).

As well as ERIC and REP sequences, repetitive BOX sequences that appear dispersed throughout the genome have also been used to subtype microorganisms. BOX elements have been shown to consist of three subunits termed boxA, boxB, and boxC. Sequence analysis revealed that boxA was highly conserved in bacteria, whilst boxB and boxC were highly variable and found only within S. pneumoniae (Versalovic et al., 1995) and since then primers for the boxA region have been used widely in several species and more recently in Aeromonas spp. (Tacao et al., 2005) and B. pseudomallei (Currie et al., 2007).

In a comparative study of molecular typing systems, Wong & Lin (2001) compared three PCR methods for subspecies typing V. parahaemolyticus that included ERIC-PCR, REP-PCR and ITS-PCR. Forty strains representing a wide range of PFGE profiles were grouped into 27, 27 and 15 patterns with discrimination indexes of 0.98, 0.97 and 0.91 by ERIC-PCR, REP-PCR and ITS-PCR respectively (Wong & Lin, 2001). In another study, PFGE was shown to be a more discriminatory method than BOX-PCR although both methods were able to distinguish between closely related strains of E. coli. However, neither of the methods used were effective in clustering E. coli strains according to the source of the organism (Cesaris et al., 2007).
2.7.9 Multi locus enzyme electrophoresis (MLEE)

Multi locus enzyme electrophoresis (MLEE) is a technique that examines the electrophoretic mobility of a selection of metabolic enzymes. The variation in mobility is visualised by staining of gels to reveal specific enzyme activity and is attributed to isozymes or allozymes. MLEE is commonly used to study long-term epidemiology on a global scale where minor differences between related strains are not required.

Farfan et al. (2000) used MLEE to study genetic relationships between a diverse group of 107 clinical and environmental isolates of \textit{V. cholerae} to determine allelic variation in 15 housekeeping enzyme loci. They identified 99 electrophoretic types with a genotypic diversity ($G$) of 0.9872 for all samples. All 15 enzyme loci studied were polymorphic, the number of alleles ranging from 2 to 7. The authors concluded that the O139 and O1 El Tor isolates were genetically more related to each other than all the other sub-populations studied.

Another study using 62 strains of \textit{V. vulnificus} strains, placed the population into two divisions that were comparable to the two divisions obtained using RAPD (section 2.7.4) (Gutacker et al., 2003). The method did not show any significant clustering or association between distinct clones and as such its use is inappropriate for subtyping isolates from local outbreaks of infection.

2.7.10 Multi locus sequence typing (MLST)

Multi-locus sequence typing (MLST) is a nucleotide sequence based approach that was proposed in 1998, borrowing from the concepts of MLEE (Maiden et al., 1998). Instead of using the variation in electrophoretic mobility of a selection of enzymes to assess allelic genotypes, the variation of the gene sequences, is bioinformatically assessed for relatedness using software and database approaches.
In a recent finding using three housekeeping gene loci and three virulence gene loci, Kotetishvili et al., (2003) demonstrated a better discriminatory ability of MLST than PFGE and since then several workers have used MLST for subtyping of *V. cholerae* (Lee et al., 2006; Danin-Poleg et al., 2007). Using MLST sequence data from six housekeeping genes of *V. vulnificus*, Cohen et al. (2007) were again able to subtype 94% of the isolates into the two distinct lineages commonly associated in this species. To date, ten housekeeping genes have been proposed for *V. vulnificus* (Bisharat et al., 2007a; 2007b). Of these ten, five of these loci have also been adopted for the MLST typing of *V. parahaemolyticus* with an additional two housekeeping loci, *recA*, *dnaE* and the variable gene *ompK* (González-Escalona et al., 2008). Using MLST of 100 isolates of *V. parahaemolyticus*, three major clonal complexes and 62 sequence types were identified. Based on these initial studies it was concluded that *V. parahaemolyticus* is genetically diverse exhibiting a semi-clonal population structure that is driven primarily by frequent recombination rather than mutation events (González-Escalona et al., 2008). However, as with all MLST methods, it is considerably more resource intensive, expensive and impractical for typing a large number of *V. parahaemolyticus* isolates from shellfish samples.

### 2.7.11 Variable number tandem repeats PCR (VNTR-PCR)

Short DNA sequence motifs that occur in specific regions of a genome where there are variations in the number of repeat units can provide a source of high genomic polymorphism are often termed variable number tandem repeats (VNTR), single sequence repeats (SSR) or 'multilocus variable number of tandem repeat analysis' (MLVA) (van Belkum, 1999; 2007). The number of repeat units that occur at a locus can vary between strains (and within strains) due to many reasons including slipped strand mispairing, deletions and insertions. VNTR analysis makes use of PCR primers complementary to the stable flanking sequences and polymorphisms in the numbers of repeat units are
detennined by agarose gel electrophoresis (van Belkum et al., 1998). In MLVA, the
approach can be supported or extended by locus-specific DNA sequencing.

VNTR has been used for a variety of applications including human genetics and bacterial
epidemiological studies most notably in subtyping Mycobacterium spp. (Roring et al.,
2004). More recently, it has been applied to members of the Vibrioneaeae; V. cholerae and
V. vulnificus. Danin-Poleg et al. (2007) screened the V. cholerae genome sequence to
reveal thousands of SSR tracts with an average frequency of one SSR every 152 bp. Using
32 strains of V. cholerae that represented both clinical and environmental isolates, the
workers tested for polymorphism in SSR loci with two methods including polymorphism
of SSR tracts longer than 12 bp and mononucleotide repeats as assessed by sequencing.
Phylogenetic analysis of combined data was able to discriminate between the clinical O1
and O139 serogroups and environmental isolates. This ability to discriminate between all
but three clinical and environmental isolates from a total of 45 strains V. cholerae was also
demonstrated (Danin-Poleg et al., 2007).

Unlike the various other PCR methods previously described, VNTR regions are sequence
specific for each bacterial species and may occur at only a single locus. Whilst VNTRs
have been widely used on variety of applications, until recently, there had been no reports
of the technique applied to V. parahaemolyticus. Kimura et al. (2008) recently showed
MLVA to have high resolution and reproducibility for typing of V. parahaemolyticus
O3:K6 that were clonally related. In 28 pandemic V. parahaemolyticus O3:K6 isolates
from human cases, MLVA produced 28 distinct VNTR patterns at eight loci (Kimura et al.
2008) and differentiated a further six environmental O3:K6 isolates that apparently showed
the same PFGE pattern. However, the parameters that were used to obtain the PFGE
clusters were unclear, and three clinical isolates were untypeable by PFGE. As with
MLST, analysis of sequence data for a large number of environmental isolates is impractical and would be difficult to implement for routine use.
Chapter Three

GENERAL MATERIALS AND METHODS

3.1 Chemicals and reagents

Unless otherwise stated all general laboratory chemicals were purchased from Amersham Biosciences, Bio-Rad Laboratories, Invitrogen, Thermo-Fisher Scientific, or Sigma-Aldrich, and, if possible, were of molecular biology grade. All bacteriological culture media was obtained from Oxoid and PCR related reagents and restriction enzymes were obtained from Roche Biosciences. Recipes for all of the buffers that were used and the suppliers’ details are outlined in Appendix I. Details of specific reagents can be found in the Materials and Methods section of individual chapters.

3.2 Bacteriology

3.2.1 Isolates of *V. parahaemolyticus*

The designation codes of *V. parahaemolyticus* obtained from various locations between 2003 and 2006 and related organisms used in this study together with their countries of origin and source of isolation where known, and PCR data for *tdh, trh* and *toxR* where available are listed in Table 3.1. A total of 86 different isolates of *V. parahaemolyticus* and closely related members of the *Vibrionaceae* were used for various typing procedures. Unless otherwise stated, the isolates were held as 50% glycerol stocks (-70°C) or on 2% Marine Salts Agar (MSA) in the University of Plymouth culture collection. A selection of biochemical tests and growth characteristics on several bacteriological media were performed regularly to confirm identity and purity of the cultures. Together with PCR and hybridisation analysis, molecular typing was performed on isolates as appropriate for which further details are provided in each chapter. These techniques included the analysis of one or more of the following: pulsed field gel electrophoresis (Chapter 5), 16S-23S and
23S-5S rRNA intergenic spacer analysis (Chapter 6), tDNA intergenic length polymorphism analysis (chapter 7) and randomly amplified polymorphic DNA (chapter 8).

The characterisation of *V. parahaemolyticus* was the first of its kind in the UK and sourcing of clinical isolates from UK bacterial culture collections proved to be very difficult. Where possible, clinical isolates were sought from other countries and this study represents a preliminary study to assess the feasibility of molecular typing techniques to determine clinical and environmental isolates of *V. parahaemolyticus*.

### 3.2.2 Culture and growth of *V. parahaemolyticus*

All *V. parahaemolyticus* isolates were recovered from 50% glycerol stocks or grown from MSA slopes stored at room temperature in the dark. Isolates were cultured for 8 to 18 hours (depending on use) at 37°C in either 15 ml volumes of tryptone soya broth (TSB) with a final NaCl concentration of 3%, or MSA with a final salt concentration of 2%, shaking in a water bath (Clifton NE5-28D) at 70 rpm.

### 3.2.3 Culture and growth of additional bacterial species

Bacterial isolates other than *V. parahaemolyticus* that were used in this study are listed in Table 2.3 together with growth medium. All halophilic *Vibrio* spp. were grown in TSB and MSA supplemented with the appropriate amount of NaCl. *Escherichia coli* was grown on Luria-Bertani (LB) medium overnight at 37°C.
Table 3.1: Description of *V. parahaemolyticus* and their genotypes, where known, that were used in this study. The Strain ID is used throughout the study.

<table>
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<th>Strain ID</th>
<th>CEFAS Reference</th>
<th>Institute(I)</th>
<th>Location</th>
<th>Environmental/Clinical/Reference strain</th>
<th>PCR (2) toxR</th>
<th>tdi</th>
<th>trh</th>
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<td>Environmental (S)</td>
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(+) Very faint amplicon in PCR
(1) Isolates obtained from the following institutions:
Centre for Environment, Fisheries and Aquaculture Science (CEFAS)
Health Protection Agency (HPA), formerly Public Health Laboratory Service, (PHLS)
Kyoto University Medical School (KUMS)
Norwegian School of Veterinary Science (NSVS)

(2) PCR for *V. parahaemolyticus* species specific toxR, and virulence tdh and trh genes (Table 3.2)
Ure: Urease test. Data provided by S. Wagley, CEFAS Weymouth Laboratories.

(3) Identity of isolate 6316 as *V. parahaemolyticus* uncertain: Chapters 6 and 7.

**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), P: *Cancer pagurus* (edible crab), E: *Mytilus edulis* (blue mussel)

### Table 3.2: Sequences of previously described PCR primers for toxR, and putative *V. parahaemolyticus* virulence genes tdh and trh

<table>
<thead>
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<th>Target gene</th>
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<td></td>
<td>F: GCGCTAAAATGGTTAAGCG R: CATTTTCCGCTTCTCATAAGC</td>
<td>Tada et al., (1992)</td>
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Table 3.3: Description of additional bacterial strains, DNA, and their genotypes, where known, that were used in this study

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<th>Species / DNA</th>
<th>Environmental/Clinical/Reference strain</th>
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<th>Ure</th>
<th>PCR (2) tdh</th>
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<td>Amersham</td>
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<td>P. aeruginosa</td>
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(1) Isolates obtained from the following institutions:
Centre for Environment, Fisheries and Aquaculture Science (CEFAS)
Health Protection Agency, formerly Public Health Laboratory Service, (PHLS)
Kyoto University Medical School (KUMS)
University of Plymouth Culture Collection (UoP)
Amersham Biosciences: Lyophilised DNA

(2) PCR for V. parahaemolyticus species specific toxR, and virulence tdh and trh genes (Table 3.2)
Ure: Urease test. PCR data provided by S. Wagley, CEFAS Weymouth Laboratories.

(3) Isolate Vib4, together with Vib1 – Vib10 (Table 3.1) originally obtained as a collection of unspeciated isolates. Whereas Vib1 – Vib3 and Vib5 to Vib10 were later confirmed to be V. parahaemolyticus, Vib4 remained unspeciated
3.3 Bacterial genomic DNA extraction

3.3.1 Cell lysis and protein precipitation

Genomic DNA was isolated from *V. parahaemolyticus* and other bacterial species using the ‘Puregene D-6000 DNA isolation Kit’ according to the manufacturer’s instructions for Gram-negative bacteria (Gentra Systems Inc., ‘DNA Isolation Kit Instruction Manual’, pages 54-57). A 10ml overnight culture of the organism was placed into a 15ml Falcon tube, centrifuged (1000 x g, 10min, 4°C) and the cell pellet resuspended in 3ml cell lysis solution with gentle pipetting. Samples were incubated at 80°C for 5 min to lyse cells. Rnase A solution (15μl) was then added to the cell lysate before incubating the samples at 37°C for 1 hour. Following this, samples were cooled to room temperature (20°C) and 1 ml of protein precipitation solution added to the cell lysate and tubes vortexed vigorously at high speed for 20 seconds to mix the protein precipitation solution evenly with the cell lysate. The samples were kept on ice for 5 min before being centrifuged (2000 x g, 10 min, 4°C).

3.3.2 DNA precipitation and hydration

The supernatant containing the DNA was poured into a clean 15 ml centrifuge tube containing a volume of isopropanol (3 ml) and mixed by inverting the tubes several times to precipitate the DNA followed by centrifugation (2000 x g, 3 min, RT). The supernatant was discarded and the tubes left to drain over clean absorbent paper. Ethanol (70%, 3 ml) was used to wash the pellet of DNA before re-centrifugation (2000 x g, 1min, RT). Again, the supernatant was discarded, tubes drained and allowed to air dry for an hour. An appropriate volume (50 - 100 μl) of DNA hydration solution (containing TE buffer) was added, depending on the amount of DNA visible upon precipitation, to dissolve the DNA. Samples were centrifuged briefly, transferred to 1.5ml microfuge tubes and incubated for 1 hour at 65° to rehydrate the DNA. Samples were mixed and briefly centrifuged again before storage at -20°C.
3.4 DNA quantification

The quality and quantity of genomic DNA was established by agarose gel electrophoresis (Section 3.8). Images of each agarose gel were captured with a gel documentation system (UViTec, UK) and the DNA concentration was estimated for each isolate by comparison with a range of the following known amounts of λ DNA standards: 0.552 µg, 0.276 µg, 0.138 µg and 0.069 µg using UV photo software (UViTec, UK). Genomic DNA was also quantified with a UV spectrophotomer (NanoDrop 100).

3.5 Primer design for PCR

Unless stated otherwise, primers for PCR reactions were designed using the aid of DNAsis for Windows software (Hitachi) and Amplify based on a Mac platform (Engels, 1993). Primers consisting of 24 bases were generated on the basis of a 50% G+C ratio and relative proximity to the desired target sequence. The presence of 2-3 guanine or cytosine bases at the 3’ end of the primer, a lack of secondary structure and primer-dimer formation was assessed using Amplify. Primers were synthesised by Sigma-Genosys.

3.5.1 Other PCR primers

Primers used for detection of V. parahaemolyticus virulence genes, tdh, trh and toxR have been described previously, (Tada et al., 1992; Kim et al., 1999) (Table 3.2). Other primers used in the current work are described in detail in individual chapter and summarised in Table 3.4.
Table 3.4: PCR primers used in this study. Specific details are provided in each chapter.

<table>
<thead>
<tr>
<th>Target regions / Method</th>
<th>Designation</th>
<th>Sequence (5' to 3')</th>
<th>Reference</th>
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<td>Maeda et al., 2000</td>
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<td>VINTR</td>
<td>TCCTTCATCAGGCTGACTG</td>
<td>Chapter 6</td>
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<td>ITS2</td>
<td>S2F</td>
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<td>GGGGGTCGAATTCCCGGCGGCCA</td>
<td>Welsh &amp; McClelland, 1991a; 1992</td>
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</tbody>
</table>

3.6 Standard Polymerase Chain Reaction (PCR)

All PCR reactions were prepared within a PCR cabinet and performed with either a MWG Primus 96 Plus (MWG Biotech) or a Perkin Elmer (TC1) thermal cycler and based on a standard reaction mixture as follows. Each reaction mixture contained 1 unit (U) of Taq polymerase (Roche), reaction buffer containing 1.5mM MgCl₂ (Roche), 24 pmol of each primer, each deoxynucleoside triphosphate (dNTP) at a concentration of 0.2mM, between 2 – 10 ng of bacterial DNA and the reaction volume was made up to 25 μl or 50 μl using molecular biology grade water (Sigma). Negative controls were set up in exactly the same way except the addition of DNA template was replaced with an equivalent volume of water. Reactions that took place in the Perkin-Elmer thermal cycler were overlaid with molecular biology grade water.
mineral oil, due to the absence of a heated lid. Various cycling conditions were used as described elsewhere. Specific details are provided in each individual chapter.

3.7 PCR product purification

PCR products were purified using the ‘High Pure PCR Product Purification Kit’ (Roche Applied Science) according to the manufacturer’s instructions. Details of the individual solutions included in this kit can be found in Appendix I. After the PCR reaction was complete the final volume of the reaction mixture was adjusted with TE buffer to 100 μl. This was transferred to a clean 1.5ml microcentrifuge tube containing 500 μl Binding Buffer (containing guanidine thiocyanate) and mixed thoroughly. The mixture was centrifuged (13,000 x g, 60 s, RT), on a column (containing the manufacturer’s filter) and flow through solution removed. The column filter tube containing PCR DNA was washed firstly with 500 μl of Wash Buffer by centrifugation (13,000 x g, 60 s, RT) and secondly with 200 μl of Wash Buffer (13,000 x g, 60 s, RT). The filter tube was reconnected to a fresh 1.5ml microcentrifuge tube and an appropriate volume (50 μl – 100 μl) of Elution Buffer added to the column before centrifugation (13,000 x g, 60 s, RT) to elute the DNA. This DNA was quantified using the procedures in sections 3.4 and 3.8.

3.8 Agarose Gel Electrophoresis

Depending upon the application, agarose gel electrophoresis was performed using 0.8% – 2% agarose made with 1 x TBE buffer. Three sizes of agarose gel were used, 30 ml, 60 ml or 250 ml volume (equating to approximately 50 x 100 mm, 80 x 100 mm and 200 x 200 mm agarose slabs). Agarose gels of 250 ml were electrophoresed at 90V for 6 h, while 60 ml gels were run for 90 min at 120V. Gels of 30 ml were run at 75V for between 20 - 60 min depending on the expected fragment sizes. A suitable volume of 6x gel loading solution (Bromophenol Blue / Glycerol, Appendix 1) was added to all samples before
loading the gels. The sample volumes and concentrations varied according to each analysis and are stated in the Material and Methods section of each chapter. The sizes of the DNA bands that were resolved on agarose gels were estimated by comparison with either 100 bp, 1 kb standards (0.3 μg) or λ ladders and these are detailed in Appendix I. Following electrophoresis, the DNA bands were stained by immersing the gel for 20 min in a solution containing 1μg of ethidium bromide (EtBr) per ml of TE buffer followed by 10 min de-stain in distilled water to remove any unbound EtBr. 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).

3.9 Restriction enzyme digests of genomic DNA

Various restriction enzymes purchased from Roche Applied Science were used for several applications. Usually, between 0.2 - 2.0 μg DNA was incubated with 10-20 units of enzyme, using the buffer supplied by the manufacturer. Digests were incubated for various periods up to overnight, at 37°C or 50°C, depending on the enzyme to ensure appropriate digestion.

3.10 Analysis of banding profiles

Banding patterns were analysed with the aid of BioNumerics (Applied Maths, Ghent, Belgium). Generally, the bands from each lane were subjected to curve calculations and analysis to exclude background noise from PCR reactions. The tracks were then normalised using two external reference positions that consisted of 100bp molecular weight markers on either side of the gel, and an internal reference; a common band that appeared in several lanes, as identified by visual inspection. Dendrograms calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using a Pearson product-moment correlation coefficient or Dice coefficient, as appropriate to
obtain the most reliable clustering patterns. BioNumerics position tolerance settings generally were 0.5% optimisation, a position tolerance of 5% of gel and ignoring faint bands. Clusters were determined based on visual inspection of dendrogram analysis. Specific details are given in each chapter.
Chapter 4

BIOCHEMICAL, GROWTH AND SEROLOGICAL VARIATION AMONGST VIBRIO PARAHAEMLYTICUS

4.1 Introduction

Bacteria have traditionally been characterised using various phenotypic procedures that include biochemical properties, antimicrobial sensitivity tests, growth characteristics on selective media, and motility. Biochemical profilling (or biotyping) has widely been used to identify *V. parahaemolyticus* at the species level. In particular, API 20E is a standardised system, used for identifying members of the *Vibrionaeceae*. Up to 21 differential characteristics have been described which show considerable variation amongst *Vibrio* spp., and in many cases resistance to antibiotics and di-isopropyl pteridine phosphate (O/129) has been used to identify *V. parahaemolyticus* from other members of this genus (Baumann, & Schubert, 1984; Elliot et al., 1995).

Using the API 20E system Martinez-Utarza and colleagues (2006) showed that clinical isolates of *V. parahaemolyticus* were correctly identified using a 0.85% NaCl diluent as specified by the manufacturer. However, environmental isolates were more correctly identified with a 2% NaCl diluent than the standard concentration. Clinical isolates grown on a 2% NaCl diluent showed non-specific profiles. Furthermore, differences in citrate (CIT), amygdalin (AMY), gelatine (GEL) and arabinose (ARA) utilisation between the two groups of strains suggest wide biochemical diversity amongst *V. parahaemolyticus*.

Environmental isolates grown *in vitro* have been shown to exhibit characteristics different from clinical and established laboratory strains, that include less growth, induction of a viable but non-culturale (VBNC) state in certain conditions and excessive polysaccharide production. There have been few published reports into the study of growth characteristics
amongst clinical and environmental isolates. Considering that clinical and environmental isolates show enhanced biochemical fitness at different salinities of 0.85% and 2% respectively, little comparison has been made of these two heterogenic groups of isolates.

To assess phenotypic traits amongst members of clinical and environmental isolates of *V. parahaemolyticus*, standard identification tests including Gram staining and oxidase tests were performed throughout the course of study. Of these, test results for cytochrome oxidase have been shown to vary amongst *V. parahaemolyticus* (Twedt *et al.*, 1969) and the absolute requirement for Na⁺ prompted some NaCl limiting growth tests. Early work also suggested a loss of viability of some cells stored in glycerol and hence viability of storage at -20°C was periodically monitored. These simple tests allowed monitoring of purity, viability, and growth characteristics on the most popular media used to cultivate *V. parahaemolyticus*. Growth was also assessed on Wagatsuma agar (WA), used principally to detect haemolysis attributed to TDH production. Finally, serotype variation amongst clinical and environmental isolates was investigated in collaboration with CEFAS Laboratories, Weymouth, UK.
4.2 Materials and Methods

4.2.1 Biochemical tests

Bacteria used in this study were Gram-stained according to standard procedures. The oxidase test was performed with oxidase strips (Fluka). Reference strains were used as appropriate. A full set of API 20E biochemical characterisation was performed in association with colleagues at CEFAS, Weymouth, UK.

4.2.2 Growth and survival characteristics

Growth of *V. parahaemolyticus* was assessed using several solid and broth media including Marine Broth (Difco Laboratories), tryptic soy broth (TSB), Wagatsuma agar (WA) and thiosulphate citrate bile salts (TCBS) agar (Oxoid). Colony morphology was examined and recorded and growth tests carried out. Briefly, growth of 20 clinical and 20 environmental isolates was monitored in broth at 0.5% and 3.0% NaCl. A 0.5% NaCl is the standard concentration of NaCl in TSB (Oxoid) whereas a 3% NaCl is widely employed for the routine culturing of *V. parahaemolyticus*. A 50 μl volume of an overnight culture grown in TSB (3% NaCl) was used to inoculate 20 ml of fresh TSB (3% NaCl) in a shaking water bath (30°C) and growth rates were determined by monitoring absorbance at 600nm. The average growth rates of clinical and environmental isolates were determined and means tested for statistical significance with a two sample t-test (NCSS software).

Assessment of viability from cold storage was made by inoculating 50μl of 50% glycerol stock stored at -20°C into 10ml of TSB (3% NaCl), after 1, 2 and 3 month intervals since initial preparation of stock culture. Cultures were grown overnight and 20μl directly streak plated onto TCBS. Viability was monitored during the first three months of setting up glycerol stocks.
4.2.3 Thermostable Direct Haemolysin (TDH) phenotype – ‘Kanagawa test’

Wagatsuma agar (WA) was prepared using fresh rabbit blood in heparin using the methods described in the FDA guidelines (Appendix 1). Briefly, fresh (within 24 h of drawing) rabbit blood was mixed with equal volume of physiological saline and centrifuged at 4000 x g at 4°C for 15 min. Supernatant was removed and the blood cells washed a further two times. After the third wash, supernatant was poured off and cells resuspended to original volume with fresh saline. Blood was used at 5% v/v with remaining agar mixture that had been steam sterilised and cooled to 50°C before use. Dried (30°C, 30 min) Wagatsuma agar plates were streak or spot plated using 5, 10 and 20 μl of overnight grown cultures of *V. parahaemolyticus*. To determine whether haemolysis was solely a result of bacterial growth on WA or heat stable haemolysin present in broth, the supernatant of the overnight grown cultures was heated to 100°C for 10 min and spot plated in 5, 10 and 20 μl WA overnight at 37°C.

4.2.4 Determination of haemolytic activity levels

Quantifying relative amounts of haemolytic activity between several strains was attempted using a modification to a method by Yoh *et al*, (1986). This method was used to potentially assess the uncertain β-haemolytic phenotypes on Wagatsuma agar. Briefly, one ml supernatant volumes of overnight cultures of test bacteria containing crude TDH were heated to 100°C for 10 min. A 50 μl volume of each of the supernatants were transferred separately to a round bottomed microtitre plate followed by the addition of 50 μl PBS containing 20 mM CaCl$_2$ and rabbit erythrocytes at a final concentration of 2% v/v. After 3 hours at 37°C the assay was performed using a microtitre plate reader (Optimax Tunable) set to $A_{540}$ using no-lysis and full lysis as relative controls.
4.2.5 K and O antigenic variants (Serotyping)

*V. parahaemolyticus* was serotyped for somatic (O) and capsular (K) antigens using a commercially available serotyping kit from Denka, Seiken Corp, Japan. using a polyvalent and monovalent anti-sera combination according to the scheme outlined in Table 4.1 and 4.2. This work was carried out in collaboration with colleagues at CEFAS Weymouth laboratory.

The K serotype was determined from the polyvalent antiserum I to IX (Table 4.1) using some modifications from the manufacturer's guidelines. Test bacteria were grown overnight on TSA supplemented with 3% NaCl and fresh colonies were densely suspended in 500 µl 3% saline. A double-welled slide, each well containing 20 µl of the polyvalent serum was used; to one well 20 µl of test bacteria was added and the other PBS (negative control). The mixture was stirred with the pipette tip and the slide tilted around to facilitate agglutination which was seen as the formation of a cloudy, granular precipitate. Only strong agglutination that occurred within one minute was considered a positive reaction. Once a polyvalent antiserum had been ascertained, the procedure was repeated with monovalent sera until a positive agglutination was achieved. Where no agglutinations took place, a new type of K antigen was presumed and was indicated by K-untypable or KUT.

Overnight cultured test bacteria as described above were suspended in 3% saline at an approximate concentration of 10 mg/ml. Glycerine at 5% w/v was used in the mixture to prevent spontaneous agglutination of *V. parahaemolyticus*. Cells were heat-lysed by autoclaving at 121°C for 15 minutes and centrifuged for 10 mins. The supernatant was discarded and the sediment used for the agglutination test. When the K type had previously been identified, the antigenic scheme in Table 4.2 was used to test for serotype. If the O antigen corresponding to the K type as shown in Table 4.2 did not agglutinate, the remaining O serum was tested.
Table 4.1: Polyvalent and monovalent antisera containing agglutinins specific to K type. Each polyvalent antisera contained a mixture of 7 agglutinins. A positive agglutination in the polyvalent sera served to narrow down and test for the monovalent K antigen (Denka Seiken Corp.).

<table>
<thead>
<tr>
<th>Polyvalent K antiserum</th>
<th>K agglutinins present</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1 3 4 5 6 7 8</td>
</tr>
<tr>
<td>II</td>
<td>9 10 11 12 13 15 17</td>
</tr>
<tr>
<td>III</td>
<td>18 19 20 21 22 23 24</td>
</tr>
<tr>
<td>IV</td>
<td>25 26 28 29 30 31 32</td>
</tr>
<tr>
<td>V</td>
<td>33 34 36 37 38 39 40</td>
</tr>
<tr>
<td>VI</td>
<td>41 42 42 44 45 46 47</td>
</tr>
<tr>
<td>VII</td>
<td>48 49 50 51 52 53 54</td>
</tr>
<tr>
<td>VIII</td>
<td>55 56 57 58 59 60 61</td>
</tr>
<tr>
<td>IX</td>
<td>63 64 65 66 67 68 69</td>
</tr>
<tr>
<td></td>
<td>70 71</td>
</tr>
</tbody>
</table>

Table 4.2: Antigenic scheme of *V. parahaemolyticus* O groups and K serotypes. K types that have been shown to correspond to O antigens to facilitate serotyping (Denka Seiken Corp.).

<table>
<thead>
<tr>
<th>O-antigen Group</th>
<th>K type¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 25 26 32 38 41 56 64 69</td>
</tr>
<tr>
<td>2</td>
<td>3 28</td>
</tr>
<tr>
<td>3</td>
<td>4 5 6 7 29 30 31 33 37 43 45 48 54 57 58 59 65</td>
</tr>
<tr>
<td>4</td>
<td>4 8 9 10 11 12 13 34 42 49 53 55 63 67 68</td>
</tr>
<tr>
<td>5</td>
<td>15 17 30 47 60 61 68</td>
</tr>
<tr>
<td>6</td>
<td>18 46</td>
</tr>
<tr>
<td>7</td>
<td>19 52</td>
</tr>
<tr>
<td>8</td>
<td>20 21 22 39 70</td>
</tr>
<tr>
<td>9</td>
<td>23 44</td>
</tr>
<tr>
<td>10</td>
<td>19 24 52 66 71</td>
</tr>
<tr>
<td>11</td>
<td>36 40 50 51 61</td>
</tr>
</tbody>
</table>

¹ Note: The antigenic scheme by Denka Seiken is different to the first established by Sakazaki *et al.*, (1963) and later revised versions (Hugh & Feeley, 1972; Kaysner & DePaola, 2004)
4.3 Results

4.3.1 Biochemical tests

Results obtained from 63 isolates of *V. parahaemolyticus* and various reference strains show Gram negative and cytochrome oxidase positive strains although 4 isolates (VP5, VP349, VP377 and VP378) showed weak blue/purple colouration of oxidase test strips even after repeat testing. Whilst all *V. alginolyticus* and *V. cholerae* isolates were positive for acid production upon sucrose utilisation as identified on TCBS agar, all *V. parahaemolyticus* isolates were negative (section 4.3.4). Isolates were confirmed as *V. parahaemolyticus* by colleagues in CEFAS based on API galleries (BioMerieux) and if they met the following criteria: positive for oxidase, negative for Voges Proskauer and Ortho-nitrophenyl-β-D-galactopyranoside (ONPG).

4.3.2 Growth rates of *V. parahaemolyticus* by reduction of Na⁺

Doubling times generated from growth curves of a selection of clinical and environmental isolates are summarised in Figure 4.1. The average doubling time (n = 20, including two references strains) for clinical isolates at a NaCl concentration of 3.0% and 0.5% were 21.1 min and 27.2 min, respectively representing a 29% increase in doubling time at the lower NaCl concentration. The average doubling time for environmental isolates at the same NaCl concentrations were 22.2 min and 31.1 min respectively, the latter this time representing a 40% increase in doubling time upon decreasing NaCl concentration (Fig. 4.1). A comparison between clinical and environmental isolates showed that at a lower Na⁺ level, environmental isolates showed more variability in growth times with less growth, as represented by a mean doubling time significantly higher (*p* < 0.001) than that of clinical isolates (Fig. 4.1) in *t*-test analysis (Appendix II).
<table>
<thead>
<tr>
<th></th>
<th>Clinical</th>
<th>Environmental</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (0.5%):</td>
<td>27.25 ± 0.62</td>
<td>31.1 ± 1.14</td>
</tr>
<tr>
<td>NaCl (3.0%):</td>
<td>21.10 ± 0.71</td>
<td>22.2 ± 0.81</td>
</tr>
</tbody>
</table>

Figure 4.1: Growth rate phenotypes of clinical (C) and environmental (E) isolates of *V. parahaemolyticus* at 0.5 and 3.0 % NaCl. The average times and error values are indicated above. Each box-and-whisker plot summarises 20 isolates grown at 30°C. Maximum and minimum doubling times outside of the inter-quartile range are indicated by error bars. Average growth rates of clinical isolates were significantly higher (*p* < 0.001 in *t*-test) than the selected environmental isolates at 0.5 % NaCl (NCSS software).
4.3.3 Culturability of *V. parahaemolyticus* isolates from -20°C

Viability of 50% glycerol stock cultures was monitored for a total of 59 isolates and this showed 28 (47%) of *V. parahaemolyticus* cultures were not culturable after a month of storage at -20°C. After another month, a further 10 (17%) isolates could not be cultured using the overnight method adopted, followed by another 5 (8%) on the third month. Out of 59 cultures, 73% of the cultures were not culturable by the third month. This included 18 clinical and 25 environmental isolates representing 100% of total clinical and 61% of total environmental isolates respectively (Table 4.3).

4.3.4 General growth characteristics on selective and non-selective media

Isolates of *V. parahaemolyticus* and related organisms (section 3.2.3) were grown on four bacteriological media and growth levels scored and recorded (Appendix II). Most organisms grew on all media at varying intensities over a period of 18 h at 37°C. *V. parahaemolyticus* grew well on both MSA and 3% NaCl TSA with more pronounced growth and larger sized colonies on MSA. Colonies appeared circular on TSA whereas growth on MSA was less raised and generally opaque, although this varied amongst members of the genus. *V. cholerae* colonies were generally more translucent on MSA and TSA. Growth on a more selective media, TCBS, usually used for isolation and confirmation purposes, generally showed less growth than MSA or TSA, yet distinct colonies of various green intensities were observed (Fig. 4.2). Unusually, amongst the *V. parahaemolyticus* cultures one clinical isolate, 6316, grew remarkably small colonies on all four types of media tested. *V. cholerae* isolates showed distinct yellow colonies upon acid production with sucrose fermentation as did *V. alginolyticus* isolates used in the study. The production of acid alters the pH and is indicated by the dyes bromothymol blue and thymol blue. The colour reverted to green within a few days.
Table 4.3: Serotypes and selected phenotypes of *V. parahemolyticus* isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Environmental/Clinical/Reference strain</th>
<th>Serotype</th>
<th>Culturability^2^ From -20°C</th>
<th>Phenotypes</th>
<th>Genotypes^4^</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Beta-</td>
<td><strong>tdh</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>haemolysis^3^</td>
<td></td>
</tr>
<tr>
<td>1902</td>
<td>NCIMB Reference</td>
<td>O1:K1</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VP10884</td>
<td>NCTC Reference</td>
<td>O3:K4</td>
<td>1</td>
<td>+++-</td>
<td>-</td>
</tr>
<tr>
<td>VP10885</td>
<td>NCTC Reference</td>
<td>O2:K3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>219</td>
<td>Clinical</td>
<td>O6:KUT</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>220</td>
<td>Clinical</td>
<td>O3:K6</td>
<td>1</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>221</td>
<td>Clinical</td>
<td>O1:KUT</td>
<td>0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>222</td>
<td>Clinical</td>
<td>O3:K6</td>
<td>0</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>223</td>
<td>Clinical</td>
<td>O3:K6</td>
<td>2</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>224</td>
<td>Clinical</td>
<td>O4:K9</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>225</td>
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<td>0</td>
<td>+++-</td>
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<tr>
<td>226</td>
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<td>O3:KUT</td>
<td>0</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>227</td>
<td>Clinical</td>
<td>O5:K68</td>
<td>1</td>
<td>+++-</td>
<td>-</td>
</tr>
<tr>
<td>228</td>
<td>Clinical</td>
<td>O11:KUT</td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1022/4</td>
<td>Environmental</td>
<td>O3:K37</td>
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</tr>
<tr>
<td>1024/5</td>
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<td>O3:KUT</td>
<td>0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1021/5</td>
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<td>O2:KUT</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1022/10</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1023/1</td>
<td>Environmental</td>
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<td>0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1023/2</td>
<td>Environmental</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>1023/3</td>
<td>Environmental</td>
<td>ND</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1103/9</td>
<td>Environmental</td>
<td>O1:K1</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2053</td>
<td>Clinical</td>
<td>O3:K6</td>
<td>0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2436</td>
<td>Clinical</td>
<td>O6:K18</td>
<td>0</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>5421</td>
<td>Clinical</td>
<td>O3:K6</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6134</td>
<td>Clinical</td>
<td>O4:K12</td>
<td>1</td>
<td>+++-</td>
<td>-</td>
</tr>
<tr>
<td>6316</td>
<td>Clinical</td>
<td>O3:K6</td>
<td>1</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Vib 1</td>
<td>Environmental</td>
<td>O1:K32</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vib 2</td>
<td>Environmental</td>
<td>O1:K32</td>
<td>0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vib 3</td>
<td>Environmental</td>
<td>O1:K32</td>
<td>2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vib 4</td>
<td>Environmental</td>
<td>O1:K32</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vib 5</td>
<td>Environmental</td>
<td>O1:K32</td>
<td>0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vib 6</td>
<td>Environmental</td>
<td>ND</td>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Vib 7</td>
<td>Environmental</td>
<td>ND</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vib 8</td>
<td>Environmental</td>
<td>ND</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vib 9</td>
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<td>ND</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vib 10</td>
<td>Environmental</td>
<td>ND</td>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VP 1</td>
<td>Environmental</td>
<td>O1:K33</td>
<td>0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VP 2</td>
<td>Environmental</td>
<td>ND</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VP 3</td>
<td>Environmental</td>
<td>ND</td>
<td>0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VP 4</td>
<td>Environmental</td>
<td>O1:K33</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VP 5</td>
<td>Environmental</td>
<td>O1:K33</td>
<td>3</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>VP 6</td>
<td>Environmental</td>
<td>O2:K28</td>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
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</table>

ND: Not determined

1 Serotyping performed in collaboration with colleagues at CEFAS. KUT: Untypeable K antigen

2 Culturability scores are:
   0 and 1 for non-viability and viability on the first month.
   2 indicates viability in the second month respectively. Viability on month 3 is recorded as 3

3 Levels of β-haemolysis was monitored at 18 hours and scored as, +++ High, ++ Medium, + Low/uncertain, - No visible haemolysis. β-haemolysis is resultant phenotype of gene *tdh* expression.

4 Potentially virulent genes: *tdh* and *trh* encoding for thermostable direct haemolysin and related haemolysin respectively.

(+) Weak amplification bands
Figure 4.1: Representative growth of *V. parahaemolyticus* clinical isolate 2436 (plate A) and environmental isolate VP4 (plate B) on TCBS agar after 18 hours at 37°C. There were no obvious growth characteristics differentiating isolates of *V. parahaemolyticus* on TCBS agar.

Figure 4.2: Representative growth of *V. parahaemolyticus* clinical isolate 2436 (plate A) and environmental isolate VP4 (plate B) on TCBS agar after 18 hours at 37°C. There were no obvious growth characteristics differentiating isolates of *V. parahaemolyticus* on TCBS agar.
Figure 4.3: Exopolysaccharide production amongst two environmental isolates of *V. parahaemolyticus*, VPEC1 (A), VPSC1 (B) and swarming phenotype of *V. alginolyticus*, VA430 (C). Isolates were grown on Wagatsuma agar for 18 hours at 37°C.
Growth on Wagatsuma agar (WA), principally used to test for Kanagawa haemolysin TDH, showed much variation as well as an unusual amount of exopolysaccharide production for two environmental isolates, VPSC1 and VP EC1, (Fig. 4.3). Of significance is that six isolates showed poor growth including NCIMB type strain 1902, clinical strain 6316, and four environmental isolates 1022/4, Vib4, VP350 and VP361. With the exception of these isolates, there appeared no obvious or distinct growth characteristics on selective or non-selective media.

4.3.5 Beta-haemolysis and clinical phenotypes

Varying degrees of haemolysis were demonstrated by isolates of *V. parahaemolyticus* and related species as indicated on Table 4.3 and in Fig. 4.4. In many cases, it was not possible to ascertain whether haemolysis had actually occurred as the zone around the colony was not characteristic of a clear β-haemolysis phenotype. Of the distinct haemolytic phenotype, all of the isolates except clinical strain 6316 were positive for *tdh*. In some cases of β-haemolysis, there appeared a secondary zone of haemolysis (Fig. 4.5) but without a green pigmentation (characteristic of α-haemolysis). This secondary zone appeared similar to the zone induced by heat treated ECPs (Fig. 4.6) and was only present in isolates that appeared to be the most haemolytic (Table 4.3).

Out of 27 *tdh* positive *V. parahaemolyticus* isolates, 12 (44%) showed distinct β-haemolysis, 10 (37%) isolates showed some signs of haemolysis, but remained unconfirmed, and a further five (19%) isolates did not exhibit any visible haemolytic activity (Table 4.3). Of these five strains, one was from a clinical isolate (5421) and four (Vib7, Vib9, VP350 and VPEC1) were environmental. The use of a spectrophotometer to assay haemolytic levels for uncertain β-haemolytic phenotypes proved to be inconclusive with the method described (Fig. 4.7).
Figure 4.4: Variation in β-haemolytic levels of rabbit erythrocytes on Wagatsuma agar after 18 hours at 37°C. Kanagawa positive strains are isolates of *V. parahaemolyticus* that express haemolysin TDH and exhibit various degrees of β-haemolysis (plates B-D, scored +, ++, +++ respectively) from the control plate A (-). Plate E shows clinical *tdh*+ isolate with an uncertain haemolytic phenotype.
Figure 4.5: Zones of haemolysis in clinical and environmental isolates of *V. parahaemolyticus*. A primary zone of β-haemolysis is followed by a larger zone of secondary incomplete (alpha) haemolysis in NCTC clinical reference strain VP10884 (A). Inoculum spot volumes of 5, 10 and 25µl of overnight TSB cultures of *V. parahaemolyticus* supplemented with 3% NaCl. Image B shows β-haemolysis attributed to an environmental strain, VP416, isolated from *Crassostrea gigas* in comparison with a non-haemolytic environmental isolate.
Figure 4.6: Zones of partial haemolysis attributed to TDH from thermostable ECP. Supernatant of overnight *V. parahaemolyticus* (isolate 6134) culture was heat-treated and spot inoculated with 5 (Q4), 10 (Q3) and 20 µl (Q2). Arrows indicate diameter of partially haemolysed zone. First quadrant (Q1), shows growth and both primary and secondary zones of haemolysis from unheated supernatant.

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Figure 4.7: Haemolytic activity of *V. parahaemolyticus*. Lane 1, Control (equal volume H₂O and RBC; Lanes 2-5; decreasing concentration of haemolysin (heat treated ECP); Lane 6, negative control (PBS & RBC). Varying haemolysin concentration had no apparent significant haemolytic effect on rabbit erythrocytes at 3 hours.
In addition to V. parahaemolyticus, there were three isolates of different species: V. cholerae (VC5), V. alginolyticus (VA430) and V. mimicus (VM499) that possessed gene tdh but exhibited no haemolysis (data not shown). Data available on the presence of the trh gene (Table 4.3) showed no association with haemolytic activity on Wagatsuma agar. Based on the current data, there did not appear to be a link between urease activity and the presence of the trh gene in V. parahaemolyticus, as both urease positive and negative isolates were present with and without the gene trh (Table 4.3).

4.3.6 Variation in O and K serotypes amongst V. parahaemolyticus

Of the 44 isolates of V. parahaemolyticus serotyped, a total of 20 different O:K serotypes were found amongst the 19 clinical, 22 environmental and three reference strains studied (Table 4.3). Amongst these isolates, six had previously been characterised as O3:K6 and were effectively used as controls and included three Norwegian and three Japanese isolates. In total, there were eight different O-types, 13 different K-types and 22 of the environmental isolates were K untypeable (KUT). Serotype O1 appeared the most common (36%) amongst all the isolates tested and was found mainly amongst the environmental group. Similarly, serotype O3 was the most common O-antigen in the clinical group (Table 4.3). Other than these two serotypes, and a larger proportion of environmental untypeable K types, there were no apparent association of O- or K- serotypes with clinical or environmental isolates. Of significance, was the identification of two UK clinical isolates (E155855 & E168143) that were of the same O3:K6 serotype associated with the pandemic genotypes.
Table 4.4: Distribution of O-antigens in isolates of *V. parahaemolyticus.* Reference strains not included in the table. A total of eight out of 13 O-types were identified.

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4.4 Discussion

4.4.1 General growth and biochemical properties

Several methods were used to study the biochemical and physical growth properties of *V. parahaemolyticus* with the aim of assessing any differences between clinical and environmental isolates. Despite the relatively small number of isolates that were available, the strains used in the study show variation in physical growth, haemolytic and serological properties. Initial confirmatory biochemical test results were consistent with the typical characteristics of *V. parahaemolyticus* (Gram negative, oxidase positive and motile). Isolates received were routinely tested for purity and assessed using these methods. Growth on several media routinely used in the isolation and characterisation of *V. parahaemolyticus* were adequate for the purposes used, although some strains (e.g., 6316 and 6134) did not grow well and others (e.g., VPEC1 and VPSC1) showed atypical growth on selected media. However, these characteristics were present in only a limited number of isolates. Based on these growth observations, there were no obvious differences between clinical and environmental isolates that would allow differentiation.
4.4.2 Growth of *V. parahaemolyticus* in reduced salinities

Salt tolerance has been used by several workers to differentiate *Vibrio* spp. (Beuchat, 1973; Palasuntheram, 1981) and to assess properties within *V. parahaemolyticus* (Twedt et al., 1969). To the author’s knowledge, there did not appear any published studies that compared growth rates between clinical and environmental isolates of *V. parahaemolyticus*. As isolates were cultured throughout the course of this present study with 3% NaCl, it was of interest to assess if a reduction in NaCl would affect growth of clinical and environmental isolates. On the basis of this present limited study, it was shown that environmental isolates grew at more variable growth rates at both 0.5 and 3.0% NaCl than clinical isolates. However, at the reduced Na⁺ level, a significantly larger proportion of environmental isolates grew slower than clinical isolates and may indicate environmental isolates are less suited for growth at reduced salinities. It would thus appear that clinical and environmental isolates of *V. parahaemolyticus* do not differentially exploit excess Na⁺ (e.g. 3% NaCl) for growth when it is freely available. For the purposes of experiments that involved culturing of *V. parahaemolyticus* isolates (e.g. tests for Kanagawa haemolysin), it would seem culturing at the higher NaCl concentration is more appropriate as this did not favour the growth of clinical or environmental isolates.

Recent work by Martinez-Urtaza and colleagues described how clinical and environmental isolates were better identified according to the API 20E profile using two different NaCl concentrations (Martinez-Urtaza et al., 2006). In addition to environmental and clinical isolates showing significant differences in biochemical tests, clinical isolates were correctly identified according to the API 20E profile using 0.85% NaCl diluent, but they presented nonspecific profiles with 2% NaCl diluent. In contrast, the use of a 2% NaCl diluent facilitated correct identification of the environmental isolates. The findings from the present study supports the work by Martinez-Urtaza and colleagues by providing evidence of better growth of clinical isolates at a lower NaCl concentration and thus a
possible explanation of more accurate API 20E profiles. Further analysis of growth rates at different NaCl concentrations would be necessary to validate these findings.

4.4.3 Culturability of cells from cold storage at -20°C

Cold temperatures have been known to induce a viable but non-culturable VBNC state in *V. parahaemolyticus* and several other species (Baffone et al., 2003). Stock glycerol cultures were prepared using a standard method (Sambrook et al., 1989) and cells monitored for survival over three months. Of some significance is that within the first month, 50% of clinical and 41% of clinical strains became non-culturable. A somewhat similar finding was made by Jiang and Chai (1996) who showed that an environmental, Kanagawa negative strain took longer to achieve non-culturability than a Kanagawa positive one. Upon observing decline of culturable cells, it was decided to keep some remaining samples of stock culture at -20°C and monitored for culturability or viability to assess any difference amongst the *V. parahaemolyticus* strains. Although tests specifically for the VBNC state of cells were not carried out here, work by Johnston and Brown (2002) showed *V. parahaemolyticus* was not inactivated at -20°C and therefore it is speculated that the *V. parahaemolyticus* strains may have entered a VBNC state. Others have shown various degrees of resuscitation of VBNC cells by treatment with catalase or sodium pyruvate (Mizunoe et al., 2000) and that resuscitation was dependent on the age of the culture and strain dependent (Wong & Wang, 2004) although this approach was not taken here.

4.4.4 Beta-haemolysis attributed to thermostable direct haemolysin (TDH)

Haemolysis on Wagatsuma agar (WA) was demonstrated by various strains of *V. parahaemolyticus*. This β-haemolysis phenotype of TDH is widely described as a marker for virulent strains of *V. parahaemolyticus* although haemolysis levels varied considerably amongst *tdh* gene positive strains tested. Of immediate interest is an environmental isolate,
VP416, recovered from oyster (C. gigas), off the Dorset coast which demonstrated remarkably strong β-haemolytic activity (Fig 3.3), appearing more haemolytic than clinically acquired isolates expressing the haemolysin TDH. As clinical isolates must originate from environmental samples, it questions whether V. parahaemolyticus TDH is expressed in C. gigas. To date, there appears little information on oyster physiology and immune status upon levels of potentially pathogenic V. parahaemolyticus.

Of the clinical isolates used in the study, approximately 55% exhibited β-haemolysis; a significantly larger proportion than that previously described amongst clinical isolates (Kelly & Stroh 1989). However, four tdh+ clinical strains (PCR data: Table 3.1) showed an uncertain β-haemolytic phenotype and such findings have also been described by others (Nair et al., 1985). Therefore, in an attempt to quantify levels of haemolysis, three strains that gave distinct phenotypes (Fig. 4.4) were assessed to see whether it was possible to assay for haemolytic activity using a simple spot plating and microtitre assay method. Previous reports have shown that TDH is able to retain 70% of its haemolytic properties after treatment at 100°C for 10 min (Nishibuchi & Kaper, 1995) and thus it was thought it may be possible to obtain relative haemolysis levels that may be used to characterise those isolates that visually showed uncertain haemolysins levels. However, both the spot plating method and the microtitre method were unable to generate discernable figures of haemolytic levels. It would appear that without concentrating TDH, a significant haemolytic reaction would not be able to be determined with a microtitre assay. Spot plating a higher volume of ECP containing TDH on Wagatsuma agar did not show any significant haemolysis either. ELISA methods have been used to quantify TDH levels (Honda et al., 1985; Yoh et al., 1995) but the difficulties and costs associated in obtaining purified TDH products mean it is not a feasible option at present for rapid quantification of TDH amongst isolates.
The large variation in TDH expressed amongst \textit{tdh}+ isolates (Kelly \& Stroh 1989; Nishibuchi \& Kaper, 1990) adds to the uncertainty of the significance of finding \textit{tdh}+ in environmental isolates. For example, several clinical isolates did not exhibit clear haemolysis, one being distinctly negative (5421), and a further two isolates that were haemolytic, showed poor growth on WA. Additionally, false positive haemolytic reactions can occur due to pH changes around the colonies, fragility of erythrocytes or haemolysins other than TDH (Nair \textit{et al.}, 1985). Such inconsistencies in the use of WA suggest it may not be a reliable method for screening environmental isolates of \textit{V. parahaemolyticus} for TDH. Despite the present findings, some workers have employed the Kanagawa test for confirmatory purposes. Cabrera-Garcia \textit{et al.}, (2004) identified four \textit{tdh}+ isolates amongst 46 environmental samples of \textit{V. parahaemolyticus} and all four isolates were shown to be Kanagawa positive. However, Kanagawa tests are not routinely performed and the use of WA may be hindered by the availability of fresh human or rabbit blood as recommended in the FDA guidelines (Kaysner \& Depaola, 2004).

In order to study actual expression of \textit{tdh} genes, the use of reverse transcriptase RT-PCR can address the issue of estimating TDH production by monitoring mRNA levels. However, putative virulence genes \textit{tdh} and \textit{trh} have been reported not to be expressed as well as housekeeping genes under certain conditions (Coutard \textit{et al.}, 2005; 2007). Others have used a transcription-reverse transcription concerted (TRC) method to quantify \textit{tdh} and \textit{trh} mRNA transcripts (Masuda \textit{et al.}, 2004; Nakaguchi \textit{et al.}, 2004), but it appears few workers who employ \textit{tdh} and \textit{trh} gene detection protocols in \textit{V. parahaemolyticus} actually studied the expression of these genes. Future developments on these methods and gene expression studies are necessary, and may provide more reliable indications as to whether the \textit{tdh} or \textit{trh} genes in environmental isolates are active and the degrees to which they are expressed.
4.4.5 Urease production and TDH-related haemolysin (TRH)

The correlation of urease production with pathogenic isolates and more importantly $trh^+$ has been reported in several studies (Suthienkul et al., 1995; Okuda et al., 1997b; Iida et al., 1997). Data obtained from previous studies on the strains tested show only five strains that are $trh^+$ and urease positive. The $trh$ primers used were based on consensus regions of $trh1$ and $trh2$ and are therefore reliable indicators of $trh$ (Tada et al., 1992). A further eight strains, seven of which were environmental, were shown to be positive for $trh$ yet did not show urease activity. Similarly, three isolates that expressed the urease phenotype lacked the $trh$ genes. Consequently, there is insufficient evidence in this study to support published data that urease production is a reliable indicator for pathogenic strains.

4.4.6 Serotyping

Serotyping is important in distinguishing the small number of strains that actually cause disease in a large number of species including *E. coli*, *V. cholerae* and *Salmonella* (reviews by Stenutz, et al., 2006; Nair, 1996; Kingsley & Baumler, 2000). The finding of a heterogeneous population of O and K antigens whilst serotyping *V. parahaemolyticus* demonstrates the wide diversity of isolates present in the study. A large number of isolates that were not K-typeable (KUT) accounted equally for approximately a fifth of the clinical and environmental isolates. The total number of different serovars (13 and 16 in clinical and environmental isolates respectively) have only two common serotypes O1:K1 and O6:K18, and other than the O3:K6 isolates amongst clinical isolates, there appear no correlation amongst the two groups of isolates. Additional environmental *V. parahaemolyticus* isolates that were serotyped do not show a predominant serotype amongst UK acquired environmental strains (Pers. Comm. S. Wagley, 2007).

The difficulty of being able to differentiate *V. parahaemolyticus* by this method is hampered because the manufacturers of the test sera (Denka Seiken, Japan) have stated that
the determination of K antigens can be affected by the presence of certain O antigens and similarly the agglutination of O type can be inhibited by K antigens.

Of all methods studied, the growth levels in different NaCl concentrations have been of most interest. The difficulty in assessing haemolytic levels of tdh expression need to be addressed if the haemolytic phenotype is to be used successfully. Although biochemical and physical growth characteristics have been explored, the relationships and ability to differentiate between clinical and environmental isolates using these approaches can be long and often difficult to interpret. Larger sample numbers and greater number of methods are required to help assess feasibility of biochemical, growth and serological characterisation. The next few chapters explore the use of molecular methods in characterising *V. parahaemolyticus*. 
Chapter 5

USE OF PULSED-FIELD GEL ELECTROPHORESIS TO SHOW CLONAL RELATIONSHIPS AMONGST VIBRIO PARAHAEMLYTICUS ISOLATES

5.1 Introduction

Pulsed-field gel electrophoresis (PFGE) is based on the observation that during continuous field electrophoresis, DNA above 30-50 kb migrates with the same mobility regardless of size. If the DNA is forced to change direction during electrophoresis, different sized fragments begin to separate from each other. PFGE thus allows separation of larger pieces of DNA than conventional agarose gel electrophoresis, and in recent years it has been used extensively to sub-type bacterial species, and is widely regarded as the method of choice for typing nosocomial and community acquired pathogens (Tenover et al., 1995). PFGE has been shown to be more discriminating than other molecular methods of typing for many species of bacteria and with V. parahaemolyticus it has been reported to have the highest discrimination index of a single typing method when compared with several other methods including ERIC-PCR, and an RFLP method based on the Fla locus (Marshall et al., 1999).

Intact genomic DNA is digested with an infrequently cutting restriction enzyme and the resulting DNA fragments are separated in agarose gels using highly specialised equipment to switch the direction of the current for different time periods. This allows the separation of DNA fragments sized between 50kb and 10 Mb. In practice, whole bacterial cells are suspended in molten agarose in small plug moulds. The solidified plug containing a known number of bacterial cells is then treated to lyse the cells in situ and remove interfering constituent proteins. The resultant linearised DNA fragments are then separated by PFGE.
Differences in the number and sizes of fragments after separation are due to variation in the positions and frequency of restriction sites on the genome (Tenover et al., 1995).

PFGE has also been used for macro restriction analysis to study entire bacterial genomes. The ability to separate large DNA fragments has enabled the identification of multiple and linear chromosomes as well as genomic reorganisation caused by duplication, mutation, insertion or deletion. Indeed, this technique has been used to unravel the complex genomic structure of several bacterial genomes and produce detailed physical maps, including that of *Vibrio parahaemolyticus* (Fonstein & Haselkorn, 1995; Kolsto, 1997; Tagomori et al., 2002; Yamaichi et al., 1999;)

The use of PFGE for subtyping *V. parahaemolyticus* was first reported in 1996 by Wong et al., (1996) and since then, several workers have developed various protocols for the application of PFGE for genomic typing of *V. parahaemolyticus* based on the initial procedures of Cantor et al., (Bag et al., 1999; Chowdhury et al., 2000a; 2004a; Lu et al., 2000; Cantor et al., 1988; Wong et al., 1996). However, variations in the use of restriction enzymes (*NotI*, *SfiI* and *ApaI*), pulse switch times, equipment and other parameters mean that PFGE patterns produced by different laboratories are not readily comparable. Moreover, in many cases variation in the degree of DNA degradation has hampered the use of PFGE sub-typing methods (Marshall et al., 1999).

The current study was initially based on the procedures described in the BioRad PFGE Kit (2001) using the electrophoresis and pulse settings of Wong et al., (2000) for *V. parahaemolyticus*. Upon the observation of large degrees of DNA degradation, several modifications were made. This work was carried out to:
(i) compare *V. parahaemolyticus* UK isolates with each other and with isolates obtained from other countries,

(ii) to assess existing methods for PFGE of *V. parahaemolyticus* for the ability to differentiate clinical (virulent) and environmental (mostly avirulent) *V. parahaemolyticus*, and

(iii) to establish a benchmark method using PFGE (described as the "gold standard" by Streulens, 1998) to which other molecular typing systems could be compared.
5.2 Materials and Methods

5.2.1 Preparation of *V. parahaemolyticus* plugs

*V. parahaemolyticus* cells grown in Marine Broth were harvested at OD$_{600}$ of between 0.8 and 1.0 corresponding to a cell density of between $10^7$ - $10^8$ cells/ml. Where possible, all reagents used were of PFGE quality from BioRad. Details of the buffers used in this section are in outlined in Appendix I. *V. parahaemolyticus* cells were embedded into agarose plugs using the method as described in the "CHEF Genomic DNA Plug Kits Instruction Manual" (CHEF Genomic DNA Pulsed Field Electrophoresis Systems - Instruction Manual and Applications guide, BioRad). *V. parahaemolyticus* cell density was established using a haemocytometer; contrast obtained by staining cells with Gram Crystal Violet. A volume corresponding to $5 \times 10^7$ bacteria was centrifuged (13,000 x g, for 3 min, RT) and the supernatant discarded. Cells were washed twice with 500μl of cell suspension buffer (CSB), and then re-suspended in 250μl of CSB and equilibrated to 50°C. Alternatively, 1.25ml from an overnight culture grown in TSB (3% NaCl) was harvested by centrifugation (without cell counting) and resuspended in 250μl of buffer containing 10mM Tris, 100mM EDTA and 1mM NaCl (pH 8.0) (Wong et al., 1996). Next a 250μl volume of molten solution of 2% (w/v) 'Pulsed-field CleanCut agarose' (50°C) was added and mixed gently. The mixture was transferred into 100μl disposable plug moulds and left to solidify at 4°C for 15 mins. Plugs were also prepared with the same method for selected isolates of *V. cholerae*.

5.2.2 Bacterial cell lysis and de-proteination

Solidified plugs were removed from plug moulds and transferred to sterile plastic disposable bottles containing 50μl Lysozyme stock and 1.25ml of Lysozyme Buffer for each 500μl of agarose plugs. Plugs were incubated for 2 hours at 37°C, after which the lysozyme solution was discarded and the plugs were washed briefly with 10ml sterile
water. Plugs were then incubated for 24 h with a proteolytic solution containing 1.25ml of Proteinase K Reaction Buffer and 50µl Proteinase K stock per 500µl agarose plugs at 50°C without agitation. Afterwards, proteinase K solution was removed and the plugs washed four times in 1x Wash Buffer, 1 hour each wash at room temperature with gentle agitation. For each plug 1ml of 1x Wash Buffer was used. During the third wash a final concentration of 1mM PMSF was used to inactivate residual Proteinase K. If undigested DNA plugs were required, then no further procedures were performed and the plugs were stored at 4°C.

5.2.3 Restriction endonuclease digestion of agarose plugs

Prior to restriction digestion, plugs were cut (4 x 9 x 1.2 mm approx) and washed for 1 hour in 1ml 0.1x Wash Buffer per 100 µl plug in a 1.5 ml microfuge tube. This was followed by a second wash (30 min) using 300 µl of 0.1x Wash Buffer. Wash Buffer solution was removed and 100µl of the appropriate restriction enzyme buffer added and left at 4°C for 30 min. Restriction enzyme buffer was removed and replaced with fresh restriction enzyme buffer and 20U of the enzyme. The contents were gently mixed and incubated overnight at the appropriate temperature.

5.2.4 Agarose gels and running conditions

Agarose gels were cast using pulsed-field certified agarose in BioRad’s gel casting kit and run at 14°C using a CHEF DR-II machine, incorporating a cooling system and buffer pump. Agarose gel concentration, switch times, running conditions and other parameters were initially based on work by Wong et al., (2000) and modified as appropriate and are included in the legends of each figure. Following electrophoresis, all PFGE gels were immersed for 30 min in 250 ml of ethidium bromide solution at 1 µg/ml to facilitate visualisation of DNA under UV illumination.
5.2.5 Standards and control organisms

A commercially available λ standard was used (Bio-Rad Laboratories) to size fragments throughout the experiments. These 48.5 kb concatemers were preparations of λ cl85sam7 ranging between approximately 0.05 and 1 mb in size. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* standards supplied by BioRad were used as controls and size markers. *V. parahaemolyticus* NCIMB reference strain 1902 was used to optimise conditions. Internal controls (DNA of same isolates) were placed at various gel lanes to assist data analysis.

5.2.6 Data analysis and dendrogram construction

DNA bands were initially compared by visual inspection and interpreted according to Tenover *et al.*, (1995). Based on the number of DNA fragments that exhibited different pulse patterns, the isolates were considered to be indistinguishable (identical pulse patterns), closely related (two to three fragments with different patterns), or possibly related (four to six fragments with different patterns). If more than six DNA fragments exhibited a different migration pattern, the isolates were considered to be unrelated. A dendrogram showing relationships amongst the clinical and environmental isolates of *V. parahaemolyticus* that appeared typeable were generated using BioNumerics Software with modules for Fingerprints and Cluster Analysis (version 5.1, Applied Maths, Belgium) as described in section 3.10, with data normalised using internal (same isolates placed at different gel lanes) and external controls (λ ladders). Dendrogram was calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using the Dice coefficient. BioNumerics position tolerance settings were 5% optimisation, a position tolerance of 2% with all visible bands scored. Pulse Types (PTs) were assigned at a cut off point of 88% similarity value that was based on visual inspection of identical and closely related isolates as described above.
5.3 Results

5.3.1 PFGE of *V. parahaemolyticus*

PFGE was performed on a total of 61 isolates of *V. parahaemolyticus* and 11 related species using restriction enzymes *NotI* and to a lesser extent with *SfiI*. The data reflect the wide genetic heterogeneity amongst the isolates used in the study (Figs. 5.1 to 5.8) and many isolates were untypeable due to excessive amount of DNA degradation. In total, 13 of the environmental isolates and one clinical isolate were not typeable using the methods described. Using the BioRad method, visible PFGE bands were present at cell densities greater than $10^9$ cells and various degrees of DNA degradation, including that of controls, *S. cerevisiae* and *S. pombe* visible throughout the gels (Figs. 5.1 & 5.2).

5.3.2 PFGE of *V. cholerae*

*V. cholerae* plugs prepared using the same methods, were digested with both *SfiI* and *NotI*. Using the same electrophoretic parameters as those used with *V. parahaemolyticus*, *V. cholerae* showed distinct banding profiles (Fig. 5.2). Only two strains of *V. cholerae*, VC1 and VC BAD01 could be typed and subsequently used for comparative purposes. *NotI* digested plugs of *V. cholerae* (VC4 & VC1) showed up to five common bands in a total of approximately 15 bands with *V. parahaemolyticus* (Figs. 5.2 & 5.8).

5.3.3 *SfiI* digested restriction profiles of *V. parahaemolyticus*

Up to 14 bands were visible with NCIMB type strain 1902 that ranged in size between 50 kb and 430 kb. A larger, unresolved, faint band (>600kb) and several smaller bands (<48.5kb), although visible, were very faint amongst other widely distributed sizes of restriction fragments (Figs. 5.2 & 5.3). The appearance of these faint bands with *SfiI* digestion in comparison with *NotI* restriction bands initially favoured the use of a *NotI* restriction profiling and therefore *NotI* was used on the entire collection of *V.
parahaemolyticus isolates available. Isolates that were not typeable using NotI also appeared untypeable using SfiI.

5.3.4 NotI digested restriction patterns of V. parahaemolyticus

NotI digested PFGE profiles showed between 11 - 18 bands that ranged in size between 30 kb to greater than 600 kb and was therefore selected for use on the entire collection of isolates available. Of the 47 typeable isolates, i.e. those that showed visible bands even within DNA smearing, 19 were of clinical origin (including two reference strains) and 28 were environmentally acquired strains. These represent 95% and 68% typeability of clinical and environmental isolates respectively using the methods described.

Environmental isolates used in the study showed a wide variation in banding profiles and included many unique restriction patterns. For example, at least seven environmental isolates were single members of their own Pulse Types (PT) and could not be grouped based on common bands (Figs. 5.5 – 5.10). The environmental isolates (n=28) were identified as 17 unique PFGE Types found distributed throughout dendrogram (Fig. 5.11). Seven of these groups consisted of between two to four isolates per Pulse Type that was usually from the same source of origin. For example, Vib1, Vib2 and Vib3, all of serotype O1:K32 from Southampton appeared to be clonally related as did Vib7 to Vib10 (Figs. 5.8 & 5.9). Similarly, the homology of VPEC1 and VPSCI (Fig. 5. 11) could be attributed to the same source of isolation (Portland, Dorset). However, between all the environmental groups, although several bands were common, there was no band that was universally present amongst all the strains, thus reflecting a true heterogeneous population of V. parahaemolyticus in this study (Fig. 5.11).
PFGE profiles generated by clinical strains of Pulse Types 18 to 22 exhibited the most similar banding patterns with at least 8 common bands out of approximately 15 bands (>80% similarity) (Figs. 5.4 – 5.8 & 5.10). This group of 10 isolates (Fig. 5.11) fell into one hierarchical cluster and included two strains obtained from the UK that had almost identical PFGE profiles to strains from Japan and Norway. These clinical isolates were of serovars O3:K6, O3:KUT and O5:K68 and were all positive for tdh and did not contain trh genes. Clinical isolates that were in Pulse Types 15 and 22, however, contained trh only strains. Additionally, an environmentally acquired tdh positive strain (VP416) showed a near identical match to a clinical Japanese strain, 6134. Norwegian isolate 221 showed the least number of bands amongst the clinical isolates with 11 fragments that were widely dispersed in fragment sizes and was thus used as a size marker for dendrogram analysis.

5.3.5 Dendrogram analysis of NotI restriction PFGE patterns
Cluster analysis based on UPGMA and a cut off point at 88% banding similarity, as generated using BioNumerics, the isolates used in the study were grouped into a total of 30 NotI restriction PTs (Fig. 5.11). Of these types, only one type, (PT 09) had a mixture of both an environmental and clinical strain in the group at 88% similarity. Isolate VP416, a UK shellfish acquired, Kanagawa positive strain matched the profile of a Japanese clinically sourced Kanagawa positive strain. Other environmental isolates were also grouped with clinical isolates but appeared to be more distant (<88% similarity) on the dendrogram. Seven clinical isolates, (PTs 02, 06, 15, 19, 24, 25 & 27) consisted of unique PFGE types and included the UK reference strain, 1902 (Fig. 5.11). Other than these strains, the clinical isolates used in the study generally clustered into one group of the dendrogram (PTs 18 - 22) with distinct clonality in PT 18. The environmental isolates exhibiting clonality were clustered in PTs 03 (Vib1 - Vib3), 04 (Vib7-Vib10), 05 (VPEC1 & VPSC1), 10 (VP1, VP2 & Vib5), 12 (VP4 & VP5) and 30 (VP6 & VP7) although
isolates in Pulse Types 28 – 30 appeared more closely related than that indicated by the
dendrogram (Fig 5.11).
Figure 5.1: PFGE profiles of restriction Not1 digested and undigested *V. parahaemolyticus* of various cell densities and treatments using BioRad method. Lanes 1 – 12 are NCIMB type strain 1902 treated with (Lanes 1-6) and without (Lanes 7-12) PMSF. Lanes 1, 2, 7 & 8 correspond to $10^8$ cells; lanes 3, 4, 9 & 10 correspond to $10^7$ cells; lanes 5, 6, 11 & 12 correspond to $10^6$ cells; lane 13: *S. cerevisiae*; lane 14: *S. pombe*; lanes 15 & 16: undigested 1902; lanes 17 & 18: environmental isolate Vib 3; M: λ ladders size indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 18 h.
Figure 5.2: PFGE profiles of NotI-digested environmental isolate V. parahaemolyticus VP1 and V. cholerae isolate VC4 with cell densities of 10^9 (A), 5x10^8 (B) and 10^8 cells (C). Lanes 1 - 7, 14 & 15: Isolate VP1; lanes 8 - 13, 17 & 18: Isolate VC4. NotI-digested are indicated by N and SfI-digested indicated by S above. Lanes 1 to 18 represent plugs prepared as replicates. Lanes 1 - 7, 14 & 15: Isolate VP1; lanes 8 - 13, 17 & 18: Isolate VC4. NotI-digested are indicated by N and SfI-digested indicated by S above. Lanes 1 to 18 represent plugs prepared as replicates.

Following conditions: 20 min pulse at 65 volts (1.9v/cm) for 20 h.

M: Ladders, sizes indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse at 65 volts (1.9v/cm) for 20 h.

M: Ladders, sizes indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse at 65 volts (1.9v/cm) for 20 h.
Figure 5.3: PFGE profiles of Cla1, Not1 and Sfi1 restriction digested *V. parahaemolyticus* NCIMB type strain1902 with and without PMSF treatment and modification of method based on Wong *et al.* (2000). Lanes 1 - 6: Cla1 digests with (lanes 3 & 4) and without (lanes 1 & 2) PMSF. Lanes 5 & 6 correspond to doubling restriction enzyme concentration (30u). Lanes 7 - 10: Not1 digests with (lanes 9 & 10) and without (lanes 7 & 8) PMSF treatment. Lanes 11 - 14: Sfi1 digests with (lanes 13 & 14) and without (lanes 11 & 12) PMSF. *S. cerevisiae* digested with Not1 treated with (lane 16) and without (lane 17) PMSF. Lane 18: undigested VP1902. M: λ ladders indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 18 h.
Figure 5.4: Comparison of PFGE profiles of NotI restriction digested *V. parahaemolyticus* clinical and environmental isolates. Lanes 1-6 correspond to the following isolates: VP2a, VP2b, E154482a, E154482b, Vib 6a and Vib6b respectively. Letters a & b indicate OD_{600} of 0.4 and 0.5 respectively after overnight growth in TSB. Lanes 8 – 10 correspond to isolates VP2, VP7 and Vib 6 respectively, prepared with plugs that were 7 days old. M: λ ladders indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 20 h.
Figure 5.5: Comparison of PFGE profiles of Not1 restriction digested \textit{V. parahaemolyticus} clinical and environmental isolates of UK origin. Lanes 1-10 correspond to the following isolates: VP3a, VP3b, VP6a, VP6b, VP7a, VP7b, E155855a, E168143a, E168143b and E155855b respectively. Letters a and b indicate OD$_{600}$ of 0.4 and 0.5 respectively after overnight growth in TSB. M: \textit{\lambda} ladders indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 20 h
Figure 5.6: Comparison of PFGE profiles of NotI restriction digested *V. parahaemolyticus* clinical and environmental isolates from UK, Spain and Japan. Lanes 1-18 correspond to the following isolate designations: VP1, VP2, VP3, VP4, VP7b, Vib3, 1022/4, 2053x, 2053, 2436, 5421, 6134, 6316, E154482, E155855a, E168143a, E168143b and VP10884 respectively. Letters a and b (after isolate numbers) indicate OD₆₀₀ of 0.4 and 0.5 respectively after overnight growth in TSB. M: λ ladders with selected sizes indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 20 h.
Figure 5.7: Comparison of PFGE profiles of NotI restriction digested *V. parahaemolyticus* clinical and environmental isolates from UK and Japan. Lanes 1-17 correspond to the following isolate designations: Vib1, VP10884, 6316, VP1, VP2, VP3, VP4, VP7, Vib3, 1022/4, 2053, 2436, 5421, 6134, 6316 and E154482 respectively. Lane 18: NCTC type strain VP10884. M: λ ladders with selected sizes indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 20 h
Figure 5.8: PFGE profiles of NotI digested UK environmental and UK clinical strains of V. parahaemolyticus. Lanes 1-18 correspond to the following isolate designations: VP1, VP2, VP3, VP4, VP6, VP7, VP8, VP9, VP10, Vib3, Vib5, Vib1, Vib2, 1103/9, E154482, E155855, E168143 and VC1 respectively. Lane 14 is a Spanish isolate of V. parahaemolyticus and lane 18 corresponds to V. cholerae. M: λ ladders with selected sizes indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 20 h.
Figure 5.9: PFGE profiles of NotI digested environmental isolates of *V. parahaemolyticus* and other *Vibrio* species. Lanes 1-18 correspond to the following isolate designations: Vib2, Vib3, Vib5, Vib6, Vib7, Vib8, Vib9, Vib10, 1022/4, 1022/10, VP350, VP5, VP378, VP416, 219, 220, VCBAD01 and VM499 respectively. Lanes 15 & 16 correspond to Norwegian clinical isolates of *V. parahaemolyticus*. Lanes 17 & 18 correspond to *V. cholerae* and *V. mimicus* respectively. M: λ ladders with selected sizes indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 20 h
Figure 5.10: Comparison of NotI digested Norwegian clinical and UK environmental isolates of *V. parahaemolyticus*. Lanes 1-18 correspond to the following isolate designations: 219, 219*, 221, 221*, 222, 222*, 223, 224, 225, 226, 227, 228, VP350, VP350, VP416, VP416, VP1 and VP1 (undigested) respectively (duplicates are indicated with *). Lanes 15 & 16 correspond to Norwegian clinical isolates of *V. parahaemolyticus*. M: λ ladders with selected sizes indicated by arrows. Gel prepared with 1% agarose, 0.5xTBE and run under the following conditions: 20 min pulse, at 65 volts (1.9v/cm), for 20 h.
Figure 5.11: UPGMA dendrogram of clinical and environmental isolates of *V. parahaemolyticus*. A total of 47 isolates were typeable by PFGE. Clusters (Pulse Types) created are based on a 88% similarity value indicated by the blue line. Red dotted area indicate two isolates from UK with clonal relatedness with Japanese and Norwegian O3:K6 isolates. Environmental isolates sharing similar profiles to clinical isolates are highlighted in green and blue. Scale above banding profiles indicates approximate sizes (Mb). BioNumerics, Applied Maths, Belgium.
5.4. Discussion

This study looked at the variation in banding patterns of NotI digested PFGE profiles amongst clinical and environmental isolates of V. parahaemolyticus. The technique of PFGE has gained increasing popularity and is currently the most widely employed typing method for V. parahaemolyticus with recent reports of standardised procedures (Wong et al., 2007; Parsons et al., 2007; Kam et al., 2008). The present work described, was conducted in 2004 to establish initial relationships between the isolates used using modifications to the BioRad PFGE method and to assess whether PFGE could distinguish pathogenic from non-pathogenic isolates. It was hoped that PFGE would serve as a benchmark subtyping method to compare clusters obtained with additional typing systems.

5.4.1 Clustering of clinical isolates

PFGE analysis showed a large genomic variation amongst clinical and environmental isolates with Pulse Types 18 - 22 showing the highest similarities of all the strains. Out of 10 clinical isolates that exhibited similar banding profiles, seven were of O3:K6 serotype, one of O5:K68 serotype and two were K untypeable (KUT). Within the homogeneous O3:K6 group of isolates, PFGE demonstrated the sensitivity to discriminate with the presence or absence of between two to three bands. A Norwegian isolate of serotype O1:KUT, believed to be one of the pandemic group, showed a similar banding profile (>88% similarity) to O3:K6 strains (Figure 5.11), consistent with previous work demonstrating clonal relationships of pandemic serotypes (Chowdhury et al., 2000b; Matsumoto et al., 2000; Okura et al., 2003). The finding of UK acquired isolates to possess almost identical banding patterns to pandemic O3:K6 isolates from Japan and Norway demonstrated for the first time the presence of clonally related, more virulent pandemic isolates in the UK. These isolates were obtained from travellers arriving from India and Thailand, so it appears that infection of V. parahaemolyticus due to O3:K6 due to
consumption of indigenous shellfish in the UK have not yet been identified or tested for. This highlights inadequate surveillance measures of pathogenic *V. parahaemolyticus* in the UK that may partially be due to low numbers of sporadic *V. parahaemolyticus* infections in the UK, most of which are attributed to travel related cases, (Anon, 2006) and the self-limiting nature of the infection.

Although some clinical isolates were grouped regardless of geographical origins, others such as E154482 and the NCIMB reference strain, 1902, with Pulse Types 22 and 23 respectively, showed less relatedness, even though they were of the same O1:K1 serotype. An additional strain, environmental Spanish isolate, 1103/9 with the same O1:K1 serotype, showed yet a different Pulse Type to types 22 and 23 thereby PFGE, in this instance, was clearly able to discriminate isolates within the same serotype.

Typically, molecular differentiation is used to characterise strains that are phenotypically, biochemically or serotypically similar as described. In this study however, it was also found that within the same Pulse Field type, different serotypes were present, such as the presence of a O5:K68 strain amongst clinical strains of O3:K6 and also the presence of two serotypes in an environmental cluster, Pulse Type 05. This phenomenon was also described by Martinez-Urtaza *et al.* who showed different serotypes from the same area in Spain were included in the same PFGE cluster (Martinez-Urtaza *et al.*, 2004), thereby reinforcing the need for careful interpretation of data and the use of additional molecular characterisation to determine accurate relationships between isolates.

5.4.2 Environmental isolates showing homology to clinical isolates

PFGE was able to group two environmentally acquired strains with known clinical strains as demonstrated by Pulse Types 09, 15 and 16. An environmental isolate, VP416 that was
previously identified as a Kanagawa positive haemolytic strain, showed remarkably similar banding profiles (>94%) to a Japanese clinical isolate (6134) with serotypes O4:KUT and O4:K12 respectively. As isolate VP416 was obtained from an oyster sample from the south coast of UK in 2001, this finding may provide some evidence of the inter-continental spread of virulent strains of *V. parahaemolyticus* amongst coastal waters, and thus highlights the need for increased surveillance of shellfish samples in the UK.

In addition to isolate 6134, an environmental isolate Vib6, from Southampton, UK, also exhibited some homology to a *trh* positive clinical isolate, E154482. With a total of 17 bands, the isolates shared 14 common bands with >86% dendrogram similarity value (Fig. 5.11). As E154482 was isolated from a travel related gastroenteritis incident arriving from Thailand, the similarities of PFGE profiles to that of Vib6 may suggest the presence of UK environmental reservoirs of *V. parahaemolyticus* that may have originated from South East Asia. PFGE data with a different restriction enzyme would provide more conclusive information regarding the relatedness of these two environmental isolates used in the study. Additional sub-typing methods (chapters 6 to 8) explore these intra-species relationships further.

### 5.4.3 Typeability with PFGE

In this study, a large degree of DNA degradation was evident throughout the experiments. It was found that the use of both turbidity measurements and cell density counts to estimate DNA concentration, and the use of chloramphenicol to inhibit replication, as used by the BioRad method, was not deemed necessary. This reduced preparation time and DNA degradation. The method by Wong *et al.*, (1996) that simply used a volume of an overnight culture of *V. parahaemolyticus* was implemented; whereby the harvested cells were washed in buffer containing 10mM Tris, 100mM EDTA and 1mM NaCl (pH 8.0). This
buffer replaced the Cell Suspension Buffer (CSB) as supplied in the BioRad PFGE kit, the content of which remains publicly unknown. Interestingly, the same isolates that were not typeable with NotI restriction were also not typeable with SfiI restriction. Untypeability of environmental *V. parahaemolyticus* strains has widely been documented by several others including Marshall *et al.* (1999) and Yeung *et al.* (2002). Marshall *et al.* reported 77% typeability amongst clinical isolates whereas Yeung *et al.* found only a single isolate untypeable amongst clonally related O3:K6 isolates.

The lengthy PFGE process described in the present work required numerous procedures that required several days to complete. Preparation of plugs and their treatment inevitably introduced DNase in addition to residual nuclease activity which may not have been adequately deactivated using the methods described. Once plugs were prepared it was found that they only remained stable for a few days therefore suggesting that meaningful comparisons could only be made using freshly prepared plugs.

Recently, a number of workers have employed the ‘One Day Standardised Laboratory Protocol for Molecular Subtyping of Non-typhoidal Salmonella by PFGE’ (Pulse-Net; Centers for Disease Control, Atlanta, Ga) procedures for PFGE typing of *V. parahaemolyticus* (Martinez-Urtaza *et al.*, 2004). This method differs mainly in that an overnight culture grown on solid plate media is directly transferred to cell suspension buffer and OD adjusted to 0.48 to 0.52 before cell lysis. Immediate cell lysis with proteinase K is followed by addition of molten agarose, premixed with SDS, to prepare the plugs. In a parallel study by colleagues elsewhere, this method correctly grouped the two UK O3:K6 strains (E155855 and E168143) with clonally derived pandemic isolates from Japan, Spain, Korea and Laos (Wagley *et al.*, 2008). However, whereas the authors showed clinical isolates 2053 and 5421 in the same cluster, the BioRad method used in this study
identified them in two different pulse types (18 and 20 for isolates 2053 and 5421 respectively) at an approximately 84% similarity level. Clearly, there is an essential requirement for standardised procedures for data acquisition and analysis for the PFGE of *V. parahaemolyticus* to enable the meaningful exchange of inter-laboratory results.

### 5.4.4 Standardisation of PFGE

Despite a large degree of untypeability in the current study, PFGE has gained increasing popularity over recent years and these methods have so far yielded an increase in typeability. However, there do not appear to be many reports of this method being used extensively on environmental strains of *V. parahaemolyticus*, as it has been shown that these are more likely to be untypeable (this work; Marshall *et al.*, 1999). More recently, the CDC PulseNet have put forward a standardised protocol for the PFGE of *V. parahaemolyticus* (Parsons *et al.*, 2007). In a multicenter evaluation of this method, that incorporated 36 well characterised strains of various serotypes from different geographical regions, a high discriminatory power (34 Pulse Types in 36 strains) and typeability was shown. The study involved clinical and environmental strains. However, there was no mention of methods of data analysis and an increasing number of bands (markers) with the addition of new strains can induce errors that are compounded exponentially thereby reducing the robustness of PFGE (Call *et al.*, 2008). It remains to be seen whether this method, or others (Wong *et al.*, 2007; Kam *et al.*, 2008) that have recently been described, will gain widespread acceptance and increased typeability of environmental strains of *V. parahaemolyticus*. Ideally, a protocol that would employ the same methods and pulse conditions to type other related organisms found in the same environment would be more beneficial. As the CDC PulseNet protocol was based on the procedures of *V. cholerae* and *E. coli* O157:H7 typing, it would seem likely that *V. cholerae* could also be typed.
5.4.5 Conclusion
To date, there has been very limited published data on the detection and prevalence of \textit{V. parahaemolyticus} in the UK. This chapter presents the first known study of the characterisation of UK isolates, using modifications to a protocol initially based on commercially available PFGE kits (BioRad Laboratories). Due to the nature of the clinical symptoms of \textit{V. parahaemolyticus} infection, together with the difficulty in correctly identifying the organism, it is probable that clinical cases are currently under reported or misdiagnosed with other self-limiting gastroenteritis related illnesses.

This chapter described the use of PFGE in assessing the relationships between clinically and environmentally acquired isolates of \textit{V. parahaemolyticus}. It was found that two UK isolates from gastroenteritis cases were closely related to those of Japan and Norway as demonstrated by PFGE profiles as well as belonging to the same O3:K6 serotype. The O3:K6 serotype is widely considered a clonally derived, pandemic strain responsible for disease worldwide (Hara-Kudo \textit{et al.}, 2003; Matsumoto \textit{et al.}, 2000; Martinez-Urtaza \textit{et al.}, 2005). This together with the finding of a non pandemic, O4:KUT isolate amongst \textit{C. gigas} in British coastal waters that showed almost identical PFGE profile to a Japanese clinical isolate and presents additional evidence of clinically significant \textit{V. parahaemolyticus} in the UK and the importance of reliable typing systems for pathogenic isolates. Using PFGE, only 47 isolates could be characterised representing 77\% of the total number of isolates. This, together with the long time required to process samples, as only a limited number of samples can be processed at any one time, adds to the difficulty in the routine use of PFGE for surveillance and epidemiological purposes. Clearly, more appropriate rapid, robust methods are required to screen large numbers of environmental and shellfish samples to differentiate potentially pathogenic isolates, that can then be
subjected to PFGE analysis or other, more discriminatory, nucleotide sequence based typing systems.
Chapter 6

DIFFERENTIATION OF VIBRIO PARAHAELOLYTICUS ISOLATES BASED ON LENGTH POLYMORPHISMS OF THE 16S - 23S INTERNAL TRANSCRIBED SPACER (ITS) REGION

6.1 Introduction

Ribosomal RNA (rRNA) operons can be found in all species of bacteria with the genes usually arranged in a defined order (16S-23S-5S), often with several copies distributed throughout the genome. These highly conserved regions make them ideal for the classification, identification and typing of isolates (Abed et al., 1995; Tilsala-Timisjarvi et al., 2001; Dingman, 2004). Both the 16S rRNA gene and 5S rRNA gene have been used to establish phylogenetic relationships between a wide variety of organisms (Gazumyan et al., 1994; Yoon et al., 1997; Maiwald et al., 2000). The internal transcribed spacer (ITS1) region between 16S and 23S genes shows the most variation, both in length and sequence (Gurtler & Stanisich 1996; Dennis et al., 1998; Perez-Luz et al., 2002; Wojciech et al., 2004; Dillon et al., 2005) and this variation can be used for distinguishing between closely related bacterial species and, sometimes, between strains (Gurtler & Stanisich 1996; Leblond-Bourget et al., 1996; Daffonchio et al., 2006). In addition to this ITS1 region, the spacer region between the 23S and 5S (ITS2) may be useful for designing species-specific probes and establishing relationships between closely related species (Ji et al., 1994; Nour et al., 1998).

Sequence information of a number of V. parahaemolyticus isolates has revealed that they possess up to 11 copies of the rRNA operon (rrn) each having identical 16S rRNA genes (rrs) but with 6 spacer classes differing in size with either 1, 2 or 3 copies of each class,
(Makino et al., 2003; Gonzalez-Escalona et al., 2005b). However, a type strain isolated in 1951, varied in two of the spacer classes; one was found to be 45 bp larger and the other 15 bp larger containing genes for tRNA$_{Ala}$ and tRNA$_{Glu}$ (Maeda et al., 2000). A preliminary analysis of the spacers, demonstrated on a few clinical isolates by Gonzalez-Escalona and colleagues, differentiated clinical isolates of \textit{V. parahaemolyticus} ITS1 spacers into four groups based on two spacer classes (Gonzalez-Escalona et al., 2006a). By sequencing two clinical isolates, VpAQ and Vp1, it was further found that ITS1 regions of 40bp and 208bp, close to the 16S and 23S genes respectively, were conserved in three clinical isolates of \textit{V. parahaemolyticus}. Despite these structural similarities and variation in spacer classes, little is known about intra-species relationships between clinical and environmental isolates based on ITS1 regions. There are currently no published data assessing the feasibility of typing \textit{V. parahaemolyticus} based on variation of the ITS1 and ITS2 regions or addressing its significance as an epidemiological typing tool with this organism.

This chapter applies the use of consensus primer pairs for amplifying ITS1 regions. ITS2 regions were also amplified with primers developed using existing sequence information of \textit{V. parahaemolyticus} (Makino et al., 2003) described in section 3.5. This chapter describes the:

i) molecular typing of \textit{V. parahaemolyticus} based upon PCR amplification of ITS 1 and 2 regions

ii) the usefulness of ITS typing as a molecular typing tool for \textit{V. parahaemolyticus} isolates

iii) evaluation of the ability to discriminate clinical and environmental isolates of \textit{V. parahaemolyticus} based on ITS1 typing.
6.2 Materials and Methods

6.2.1 Primers for amplification of ITS regions

Primers VINTF (5'-TGGGGTGAAGTCGTAACAAGG-3') from 16S rRNA corresponding to *E. coli* 16S positions 1485 to 1505 and VINTR (5'-TCCTTCATCGCCTCTGACTG-3') from *E. coli* 23S positions 37 to 56 were used to amplify ITS1 regions (Maeda et al., 2000). *E. coli* 16S is notably larger than *V. parahaemolyticus* 16S: 1541bp compared to 1471bp (Brosius et al., 1978; Makino et al., 2003) and primer VINTF is complementary to a conserved region (section 6.4.4; Gonzalez-Escalona et al., 2006a) outside of *V. parahaemolyticus* 16S (Fig. 6.1). Primers for ITS2 were designed based on conserved 23S and 5S regions that flanked the ITS2 region of *V. parahaemolyticus* (Makino et al., 2003) as described in section 3.5 (Fig. 6.1). ITS2 primer sequences were designated S2F, 5'-CTAATTGCCCCGTAGGGCTTA-3' and S2R, 5'-GAGTCAGGGTGGGTCAAAAAC-3' for forward and reverse primers respectively.

![Figure 6.1: Schematic representation of the V. parahaemolyticus rRNA operon (rRNA), ITS regions and primers used in the study. Sizes are based on clinical isolate RIMD2210633 (Makino et al., 2003). Primer VINTF is complementary to a conserved region in V. parahaemolyticus ITS1.](image)

6.2.2 Amplification of ITS1 and ITS2 regions

PCR amplifications were performed as described in section 3.6 using an MWG thermo cycler (Primus 96 Plus). Conditions were optimised with the following modifications: reactions were performed in 55 µl volumes and contained 20 ng of genomic DNA and 25 pmol of each forward and reverse primer. Each reaction mixture contained 1U of Taq
polymerase (Roche), 1 x reaction buffer containing 1.5mM MgCl$_2$ (Roche) and each deoxynucleoside triphosphate (dNTP) at a final concentration of 0.2mM.

The final cycling conditions for the ITS-PCR were as follows: an initial denaturation step at 94°C for 2 minutes followed by 30 cycles of 94°C for 60 sec, 62°C for 45 sec, and 72°C for 90 sec and a final extension at 72°C for 5 min. Cycling conditions were optimised by performing PCR reactions at different annealing temperatures and with genomic DNA of three strains; type strain NCIMB 1902, Japanese clinical isolate 2053, and Spanish environmental isolate 1023/1, and PCR performed on a total of 56 isolates of *V. parahaemolyticus* including a number of replicates. Additional species that were examined included, *V. cholerae, V. vulnificus, V. mimicus* and several members of non-*Vibrio* spp. All PCR products were analysed and compared by electrophoresis of 12 µl of each reaction mixture in 2% agarose gels as described in section 3.8.

6.2.3 Data analysis and dendrogram construction

Amplicons generated with primers VINTR and VINTF were scored both empirically by means of visual inspection and by software analysis. Isolates were grouped according to fingerprint pattern types and also by using a curve based correlation coefficient and a band based Dice coefficient with BioNumerics as described in section 3. The parameters that were varied in BioNumerics during the identification of bands were “minimum profiling” (used to identify bands based on the elevation of densitometric peaks relative to the highest peak on the pattern), “minimum area” (used to identify bands on the area of densitometric features relative to the total area of the pattern) and “shoulder sensitivity” an option that allows identification of doublet bands where the curve appears as a “shoulder” to another band. BioNumerics optimisation, position tolerance settings and percentage similarity cut off points were altered in small increments until clusters resembling similar banding
profiles were achieved. A final position of 93% similarity was used for the dendrogram and ITS1 groupings.

6.3 Results

6.3.1 Consensus primers for ITS1 regions

Primers described by Maeda et al. (2000) were tested at PCR annealing temperatures of 62 - 68°C on two isolates of *V. parahaemolyticus*, NCIMB type strain 1902 and environmental isolate 1023/1 (Fig. 6.2). Bands of varying intensities were identified on the fingerprint pattern with more bright bands indicative of multiple ITS1 regions of similar sizes present in *V. parahaemolyticus* (Section 6.4.5). It was found that varying the temperatures within these ranges did not significantly alter the banding profiles with higher annealing temperatures resulting in decreased yield of PCR products (Fig. 6.2). Increasing the annealing temperature reduced background PCR noise but also reduced the intensity of amplicons generated (Fig. 6.2). However, increasing template and primer concentrations, from 10 to 20 ng and 10 to 50 pmols respectively, was shown to specifically increase the amplification of the 390 and 635 bp spacer regions (Fig. 6.2, Plate A, lanes 6-8) and was used to help deduce optimised reaction conditions (section 6.2.2). However, increasing primers and templates did not increase the intensity of the two larger amplicons of approximately 790 and 810 bps. Faint bands above 790 bps were ignored from BioNumerics analysis.

6.3.2 PCR of 23S-5S (ITS2) intergenic spacer regions

Primers that were designed based on published 23S and 5S sequences of *V. parahaemolyticus* amplified a single unambiguous band of approximately 150bp in UK type strain 1902, Spanish environmental isolate 1023/1 and clinical isolate 2053 (Fig. 6.3). Amplicons from all three isolates appeared consistent in size reflecting an ITS2 region of approximately 90 bp (without primer regions).
6.3.3 PCR of 16S-23S (ITS1) intergenic spacer regions of *V. parahaemolyticus*

PCR with primers VINTF and VINTR flanking the 16S-23S rRNA regions was carried out on 56 strains of *V. parahaemolyticus* (Figs. 6.4 to 6.8). Between four and eight bands were amplified with two distinct markers of approximately 390 and 635 bps that distinguished all but one isolate (6316) of *V. parahaemolyticus* from all the other species (Fig. 6.9). With the exception of isolate 6316, these two amplicons appeared of similar size in all of the remaining isolates tested (section 6.3.4; Fig 6.11) with some apparent variability in size of the 390 bp amplicon amongst the environmental isolates (Figs 6.4 & 6.8). Isolate 6316 was characterised by a unique profile and did not match any of the other *Vibrio* spp. tested by ITS1-PCR. In addition to the 390 and 635 bps bands, a further three bands of approximately 640, 790 and 810 bps were present in most of the isolates, with the larger sizes of bands generally appearing more faint. A further four bands, (between 390 and 640 bps, Fig. 6.10) some faint, were found distributed in some of the isolates. Based on general banding profiles, there appeared no obvious correlation of banding patterns with the source or the location of the isolates.

6.3.4 Reproducibility of ITS1-PCR banding profiles

A comparison of repeated ITS1 fingerprint profiles (Figs. 6.4 – 6.8, isolates underlined) demonstrated the degree of reproducibility of the ITS-PCR technique. A total of 17 arbitrarily chosen isolates, that included three non *Vibrio* spp. were subjected to repeat ITS1-PCR and gel runs. Five UK clinical isolates in Figure 6.6 (Lanes 16 - 20) amplified the same bands as Figure 6.5 (Lanes 12 - 14, 16 & 18). However, banding patterns, in common with the rest of the isolates on the gel, appeared more intense with more background PCR noise in Figure 6.6. Similarly, eight environmental isolates (Figs. 6.5, 6.7 & 6.8, underlined) showed reproducibility of the two main amplicons. However, identification of faint bands appeared problematic in some isolates and in some of the repeats performed faint bands could not be identified at all. For example, initial
experiments with NCIMB reference strain 1902, (Fig 6.2, Plate A, 62°C) identified two faint bands between 500 and 600 bps, both bands were absent in Figure 6.4 (Lane2) and only one present in Figure 6.6 (Lane 20). A full comparison of the bands produced in repeat analysis is shown in Table 6.1. Of the total 17 repeats, a number of the faint bands could not be ascertained in at least five isolates including 1902, VP491, VP939, VPEC1 and VPSC1 (Table 6.1).

6.3.5 ITS1 PCR in *Vibrio* species

The 16S-23S rRNA spacers in *Vibrio* species varied amongst members of the genera tested (Figs. 6.3, 6.4, 6.5 & 6.7). Using the same consensus primers, up to seven bands of various intensities were present. Amongst the six known *Vibrio* spp. tested, *V. cholerae* shared a common band of approximately 635 bp with *V. parahaemolyticus* that was present in all six isolates including *V. cholerae* NCTC type strain 8042 (Fig. 6.8). An unspeciated *Vibrio* sp., Vib4 (Table 3.3), showed a profile matching that of *Listonella damsela* LD365 (Fig. 6.5) Based on initial ITS1-PCR, *L. damsela* appeared more closely related to *V. vulnificus* than *V. cholerae* or *V. parahaemolyticus*, although a larger number of isolates and sequence analysis would be required to verify ITS1-PCR homologies between these species. Based on a limited number of isolates, the PCR developed was able to distinguish between all six *Vibrio* spp. and five non-vibrios (Fig. 6.9).

6.3.6 Dendrogram analysis and differentiation of *V. parahaemolyticus* isolates

Dendrogram analysis employing a curve based correlation coefficient was used to cluster *V. parahaemolyticus* into thirteen groups (A - M) at a 93% similarity value (Fig. 6.11). These groups appeared to be based on the positions and intensities of the two more prominent bands (approximately 390 and 635 bps) with faint bands largely unaccounted for (Fig. 6.11).
ITS types A and B consisted of a single clinical isolate, clustered with two environmental ones and a reference strain, NCIMB 1902. Isolates of these two groups appeared similar in banding profiles to those of Group C. This group, ITS-C appeared the largest with a mixture of both 32 clinical and environmental isolates and appeared closely related with VP346 (ITS Type D); the isolate differentiated based on the presence of two distinct bands between 390 and 500 bps (Fig 6.7). Most of the clinical isolates were found clustered in one of two groups amongst ITS-C with two bands around 640bp appearing as 'doublets' in comparison with environmental isolates of the same group (Figs. 6.6 & 6.11). Furthermore, two faint bands between 390 and 635 bp appeared variable amongst the 13 ITS Types. For example the 540 bp amplicon (Fig 6.10) was present in 14 out of 17 of clinical isolates whereas this amplicon could be identified anywhere in between 13 to 20 of 36 environmental isolates and thus highlighting variability in banding intensities (Section 6.3.4).

Other than isolate 6316 and reference strain VP10884, the remaining ITS Types, E – L, did not contain a mixture of both clinical and environmental isolates in any of the groups (Fig. 6.11). The most significant variation was found in the amplification of multiple ITS1 spacers of various lengths that were not identified in any of the clinical ITS types. In addition to the 390 bp amplicon, four environmental isolates, VP346, VP939, VPEC1 and VPSC1, amplified two other spacer regions of approximately 425 and 450 bps (Figs. 6.4 & 6.7). In all four isolates the 390 bp fragment was present, but was of reduced intensity. Several other isolates also exhibited a 415 amplicon, but appeared faint and unaccounted for in the dendrogram. Environmental isolates also differed by the variable size of the main spacers found in V. parahaemolyticus. These were clustered together in ITS groups E, G and H (Fig. 6.11). In addition to the new spacer types and intensity of some of the amplicons, length polymorphisms in the '390 bp' amplicon were observed. For example, isolates of E, G and H groups showed smaller amplicons than typically found in the other
groups. Similar length polymorphisms in spacers were also identified in environmental isolates of *V. parahaemolyticus*. Clinical isolates, in general, appeared more homogeneous in banding profiles.
Figure 6.3: PCR amplification of ITS2 regions of *V. parahaemolyticus* using primers designed for conserved 23S and 5S regions. Lanes 1 – 3 correspond to NCIMB type strain 1902, lanes 4 – 6; Spanish isolate 1023/1, lanes 7 – 9; Japanese isolate 2053 with decreasing template DNA concentrations of 20ng, 10ng and 5ng respectively. Lane M: 100bp ladders, sized of selected markers indicated by arrows on the right.

Figure 6.4: PCR amplification of ITS1 regions from clinical (C) and environmental (E) isolates of *V. parahaemolyticus* and other species (unmarked) using primers VINTF and VINTR. Lanes 1 – 20 correspond to the following isolates: 1: *E. coli* C1a; 2: 1902 Reference strain; 3: 2053; 4: 2436; 5: 5421; 6: 6134; 7: 6316; 8: *V. alginolyticus* VA2341; 9: Vib3; 10: Vib5; 11: VP416; 12: VP491; 13: VP939; 14: VPEC1; 15: VPSC1; 16: *V. anguillarum* CM104; 17: *S. typhimurium* ST5; 18: *K. aerogenes* KA5; 19: *P. aeruginosa* PA1; 20: *E. coli* BL21. Lane M: 100bp ladders, size of selected markers indicated by arrows on the right.
Figure 6.5: ITS1 fingerprinting patterns of clinical (C) and environmental (E) isolates of *V. parahaemolyticus* and related species (unmarked) using primers VINTF and VINTR. Lanes 1 - 20 correspond to the following isolates: 1: Negative control; 2: VP10; 3: *V. fluvialis* 11327; 4: Vib1; 5: Vib2; 6: Vib3; 7: Vib4 (unspecified *Vibrio* sp.); 8: Vib5; 9: Vib6; 10: Vib7; 11: Vib8; 12: Vib10; 13: *V. vulnificus* KIDbVV; 14: *V. vulnificus* VV11067; 15: *Listonella damsela* LD365; 16: E154482; 17: E155855; 18: E168143; 19: 2436; 20: 6134. Isolates underlined are repeat PCRs. Lane M: 100bp ladders, size of selected markers indicated by arrows on the right.

Figure 6.7: Comparison of ITS1 fingerprinting patterns of environmental isolates of *V. parahaemolyticus* from UK and Spain. Lanes 1 – 20 correspond to the following isolates:- 1: Negative control; 2: VP1; 3: VP2; 4: VP3; 5: VP4; 6: VP6; 7: VP7; 8: VP8; 9: VP9; 10: VP10; 11: 1021/5; 12: 1022/10; 13: 1023/1; 14: 1023/3; 15: 1024/5; 16: 1103/9; 17: VP331; 18: VP345; 19: VP346; 20: VP349. Isolate underlined represents a repeat PCR. Lane M: 100bp ladders, size of selected markers indicated by arrows on the right.

Figure 6.8: Comparison of ITS1 fingerprinting patterns of environmental isolates of *V. parahaemolyticus, V. cholerae* and other species. Lanes 1 -20 correspond to the following isolates:- 1: VP350; 2: VP361; 3: VP377; 4: VP378; 5: VP416; 6: VP491; 7: VP939; 8: VPEC1; 9: VPSC1; 10: VP10884 (Type Strain); 11: *V. cholerae* Reference Strain 8042; 12: VC1; 13: VC2; 14: VC3; 15: VC4; 16: VC5; 17: Proteus mirabilis PM5; 18: *S. typhimurium* ST5; 19: *K. aerogenes* KA5; 20: *P. aeruginosa* PA1. Isolates underlined are repeat PCRs. Lane M: 100bp ladders, size of selected markers indicated by arrows on the right.
Table 6.1: Reproducibility of ITS1-PCR bands in repeat tests. Tests 1 and 2 refer to independent ITS-PCR and gel runs. Uncertain band numbers are hyphenated. Corresponding band numbers in two repeats are indicated in bold. The 390 and 635 bp bands were present in all 14 isolates of *V. parahaemolyticus* in both tests. Discrepancies in the number of bands were also the result of inconsistencies in gel running conditions.

<table>
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<th>Isolate</th>
<th>No. of bands</th>
<th>References</th>
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<td>1902 (Type Strain)</td>
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<td>5</td>
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</tr>
<tr>
<td>Vib5</td>
<td>4-5</td>
<td>5</td>
<td>6.4: 10 &amp; 6.5: 8</td>
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<td>3</td>
<td>6.5: 2 &amp; 6.7: 10</td>
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<td>4</td>
<td>6.4: 11 &amp; 6.8: 5</td>
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<td>6-8</td>
<td>6.4: 14 &amp; 6.8: 8</td>
</tr>
<tr>
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<td>4-5</td>
<td>6-8</td>
<td>6.4: 15 &amp; 6.8: 9</td>
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<td><em>K. aerogenes</em>, KA5</td>
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Figure 6.9: Comparison of ITS-PCR to differentiate representative *V. parahaemolyticus* from other members of *Vibrio* spp., and related bacteria. Arrow indicates a 390 bp spacer unique to *V. parahaemolyticus*.  

<table>
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<tr>
<th>Isolate</th>
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<td><em>V. parahaemolyticus</em></td>
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<tr>
<td>VP416</td>
<td><em>V. parahaemolyticus</em></td>
</tr>
<tr>
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<td><em>V. cholerae</em></td>
</tr>
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<td><em>S. typhimurium</em></td>
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Figure 6.10: Length variations of 16S-23S (ITS1) spacers found in clinical and environmental *V. parahaemolyticus*. Lanes 1 & 3: bands found only in *V. parahaemolyticus* with bands of Lane 1 as the predominant markers for the species. Lanes 3, 4, 5, 6 & 9: Bands found in some isolates of *V. parahaemolyticus*. Bands above 790 bp were ignored. Data normalised in BioNumerics with internal and external reference positions with approximate sizes of bands (bp) indicated by arrow and below each lane.
Figure 6.11: ITS1-PCR banding profiles from 56 isolates of *V. parahaemolyticus* used in the study. The dendrogram was generated using BioNumerics and a densitometric curve based analysis with groups determined as shown with a dotted line at 93% similarity. Groupings (A - M) resulted from bias of predominant (darker) bands over faint bands. Based on this study, the identity of isolate 6316 is questioned.
6.4 Discussion

Both the length and the sequence of the ITS region separating the 16S and 23S rRNA genes are increasingly used to assess relationships in bacterial population studies (Daffonchio et al., 2006; Stewart & Cavanaugh, 2007). This chapter assessed the feasibility of ITS length polymorphisms as a simple method for sub-typing *V. parahaemolyticus* based on consensus 16S and 23S primers. The 56 isolates used in the study were assigned to 13 ITS Types, of which all but two clinical isolates fell into a single ITS Type that included an isolate (6316) initially identified as *V. parahaemolyticus*, the identity of which became questionable after ITS1-PCR analysis. Environmental isolates appeared more variable and were found included in all but two ITS Types thereby providing a simple and efficient means of differentiating isolates of *V. parahaemolyticus*. ITS1-PCR was thus shown to be a quick and reliable way of inter and intra-species differentiation of *V. parahaemolyticus*. The following discussion addresses some of the issues with the ITS1-PCR technique and highlights some further work in this area of study.

6.4.1 Primers used in the study

The consensus primers used in the present study (Maeda et al., 2000) were shown to be of identical complementary match to *V. parahaemolyticus* regions based on BLAST comparisons at all 11 operon sites of the *V. parahaemolyticus* genome based on strain RIMD 2210633 (Makino et al., 2003). Expected amplicon sizes for the sequenced strain are shown in Table 6.2. Based on these primers VINTF and VINTR successfully amplified up to eight bands despite the various degrees of background PCR noise that was present. Several approaches were taken to optimise reaction conditions and minimise the background PCR noise that was expected from the use of consensus primers in amplifying a number of products in a single PCR reaction. Bands of various intensities were produced implying either a favoured amplification of certain regions due to base mismatch in others or, as in this case, the presence of multiple amplicons of the same size (section 6.4.4).
6.4.2 ITS1-PCR banding patterns

*V. parahaemolyticus* isolates used in the study were categorised into 13 groups based on a similarity score of 93% in dendrogram analysis. This point was selected as it differentiated isolate VP346 (ITS-D) from ITS-C, with three distinct bands less than 450bp that clearly distinguished this isolate from others. Despite this prominence, there was only a marginal difference in BioNumerics cluster analysis of less than 1% between VP346 and ITS-C. However, by using a 93% dendrogram similarity, other isolates that appeared visually similar were differentiated into their own types. For example, isolates in ITS groups A and B appeared similar to those of group C but it is likely that gel intensities and background subtraction used in BioNumerics would have had some impact on reducing actual band intensities and thereby affecting calculations in the BioNumerics curve based analysis method. It is noteworthy that all four profiles of groups A and B, were from the furthermost lanes of gels where the UV illumination and the lightening system of gel photography may not have been optimal and hence background processing methods in BioNumerics may have incorrectly grouped similar banding isolates in separate clusters. This error is likely to be more apparent in techniques such as ITS1-PCR as the analysis method is reliant on the discrimination based only on a few prominent bands. RAPD analysis (Chapter 8) explores whether a more complex banding pattern can minimise any such errors.

Faint bands appeared largely unaccounted for in dendrogram analysis. Isolates in ITS-C could be visually identified as at least 4 banding types based on the presence of a ‘doublet’, and the presence or absence of faint bands between the 390 and 635bps. However, verification of these doublets would require more reliable size determination by use of acrylamide gels, or more accurately with the use of a DNA sequencer. The presence of these bands, were variable in some of the repeat tests and identification remained subjective. Despite this, and as isolates of ITS-C were from two gels (Figs. 6.6 & 6.7), the
grouping illustrates some degree of comparability between two independently run ITS1-PCR experiments. However, environmental isolates of ITS-I, J and K that showed similar banding profiles to clinical isolates of ITS-C were distinguished into their own groups. This highlights a further factor for consideration when comparing different gels: bands that appeared apparently the same by visual inspection were grouped differently on the dendrogram. To what extent this could be attributed to BioNumerics' inaccuracy in normalisation of gels or to the ITS1-PCR technique itself remains uncertain. For example longer gel runs may have been able to better resolve small differences in amplicon sizes. Inherently, the ITS-PCR method, as with most other PCR typing methods, contains variables such as temperature control and buffer quality that are difficult to control, some of which are discussed in section 6.4.3. To enable more accurate comparisons in BioNumerics, isolates should be randomly distributed amongst the gels together with an internal reference for gel normalisation to verify ITS1-PCR as a useful typing method.

6.4.3 Properties of the ITS1-PCR system

ITS1-PCR was shown to be a simple method of typing *V. parahaemolyticus* throughout the study where all isolates of *V. parahaemolyticus* appeared typeable. In some cases, individual agarose gels appeared unresolved (e.g. Figs. 6.4 & 6.8) and these are likely to be due to agarose gel running conditions and may reflect the age of buffers employed. A concentration of 2% (w/v) agarose gels was used to achieve higher resolution of bands and thus increased gel running times and higher buffer temperatures may be partially responsible for these effects. To overcome such issues and to resolve bands which essentially all lay within a 400 bp region, other workers have employed polyacrylamide gels and have targeted more variable regions (section 6.4.4) within the ITS1 domain to obtain smaller amplicons (Maeda *et al.*, 2000; Gonzalez-Escalona *et al.*, 2006).
Perhaps the most significant criticism related to the ITS1-PCR system is also the attribute that would most favour its use. Multiple ITS1 regions of the same length, especially likely in strains possessing a large number of rRNA operons, may be difficult to distinguish and thus be unaccounted for, leading to bias in interpretation and phylogenetic groupings (Boyer et al., 2001). For example, the dendrogram presented in this present work clearly shows a bias in favour of the position of the more intense bands with weaker bands largely ignored (Fig. 6.11). Although this may be useful in its own right, as it highlights the significance of two, three or more copies of the ITS1 spacer, it may not necessarily reflect true diversity of single copy spacers that may exhibit greater variability and thus more useful for a dendrogram based analysis.

To overcome the bias generated by BioNumerics, several parameters (section 6.2.3) were altered for dendrogram construction to obtain reliable groups that corresponded with similar banding profiles. A 'band based' Dice coefficient was also employed but failed to adequately group similar isolates together, most likely due to the close proximity of the bands. A recent study that assessed BioNumerics parameters for optimal correspondence of *E. coli* strains showed that a curve based method was found to be more robust and reliable than the 'band based' banding profiles in REP-PCR (Goldberg et al., 2006). Clearly, further work optimising parameters would be necessary for more accurate software based analysis. The findings from these experiments highlight the need for careful empirical assessment of groupings that are automatically generated from software based analysis.

6.4.4 Sequence based analysis of ITS1

Due to the relative large copy number of the rRNA operons present in *V. parahaemolyticus* and the presence of multiple ITS1 of the same size, sequence analysis of spacer regions was not pursued in the present study. Clearly, the source of the ITS1 variation present in some of the isolates have not been established. The 11 copies of the 16S genes of the
sequenced strain, RIMD 2210633, have been shown to possess identical nucleotide sequences (Makino et al., 2003). The design of operon specific primers would therefore involve initial ‘Long PCR’ followed by primers targeted to the conserved sequences, flanking ITS1 regions. Gonzalez-Escalona et al. (2006a) demonstrated that conserved regions of 40 bp and 209 bp, flanking the 16S and 23S regions respectively, were present in the ITS1 of \textit{V. parahaemolyticus} (Fig. 6.12). Therefore, cloning and sequence analysis of a smaller region may help to establish further relationships between clinical and environmental isolates of \textit{V. parahaemolyticus}. The findings in the present study show that the 540 and 590 bp amplicons exhibit the greatest variability in size, and thus one or both of these ITS1 regions may provide a source for genetic heterogeneity.

6.4.5 ITS1 spacer classes in \textit{V. parahaemolyticus}

The presence of multiple rRNA operons in the same genome have been widely documented in a large number of species including \textit{E. coli}, \textit{Salmonella}, \textit{V. cholerae} and \textit{V. vulnificus} (Boyer et al., 2001; Chun et al., 2002; Gonzalez-Escalona et al., 2006a). Up to 14 rRNA operons in psychrotolerant strains of \textit{Bacillus cereus} have recently been reported (Candelon et al., 2004) with operon numbers of seven commonly found in bacteria. However, approximately 40% of bacterial species are believed to have just one or two copies of the rRNA operons (Acinas et al., 2004) and in such instances it is unlikely that length polymorphisms identified with ITS1-PCR would serve as a useful tool for sub-typing.
Figure 6.12: Schematic representation of the ITS1 conserved regions in *V. parahaemolyticus*. Up to six spacer classes between 277 and 706 bps identified possessing genes for up to four tRNA (Table 6.2; Maeda et al., 2000; Gonzalez-Escalona et al., 2006a).

Table 6.2: Comparison of spacer classes identified in different *V. parahaemolyticus*. Variations in spacer sizes (*a – c*) based on four sequenced clinical strains that may be used to discriminate isolates are underlined. Column *d* shows approximate sizes based on current study.

<table>
<thead>
<tr>
<th>ITS1 Spacer Class</th>
<th>tRNA genes found in ITS1 region</th>
<th>ITS1 spacer sizes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>277 278 278 278 268-278</td>
</tr>
<tr>
<td>E</td>
<td>Glu (UUC)</td>
<td>475 476 461 431 428-478</td>
</tr>
<tr>
<td>IA</td>
<td>Ile(GAU), Ala(UGC)</td>
<td>523 524 524 524 523</td>
</tr>
<tr>
<td>AE</td>
<td>Ala(GGC), Glu(UUC)</td>
<td>541 542 542 527 528</td>
</tr>
<tr>
<td>EKV</td>
<td>Glu(UUC), Lys(UUU), Val(UAC)</td>
<td>668 669 669 669 678</td>
</tr>
<tr>
<td>EKAV</td>
<td>Glu(UUC), Lys(UUU), Ala(UGC), Val(UAC)</td>
<td>705 706 706 706 698</td>
</tr>
</tbody>
</table>

*a* Based on Maeda et al., 2000

*b* Based on two isolates, type strain ATCC17802(Aq and RIMD 2210856 (Gonzalez-Escalona, et al, 2006)

*c* Based on clinical strain RMID 2210633 (Makino et al, 2003)

*d* Estimated sizes and likely class assigned based on this study (band sizes minus 112 bp)
Based on the present limited study, it is most likely that the intensity of some of the bands (390 and 635 bps) are due to multiple copies of the same ITS spacer found in *V. parahaemolyticus* (Maeda *et al.*, 2000; Makino *et al.*, 2003; Gonzalez-Escalona *et al.*, 2005b, 2006a) although sequence analysis of representative strains would be required to confirm these findings.

Studies on the structural variation of the 16S-23S spacer regions of *V. parahaemolyticus* have identified six spacer classes based on different tRNA gene compositions (Table 6.2). These classes were based on four clinical strains two of which were of the pandemic O3:K6 serovar that are widely regarded to be clonally related (Matsumoto *et al.*, 2000; Chowdhury *et al.*, 2000b). Furthermore, ITS1 lengths between all four strains were in close agreement with each other (Table 6.2) with the lengths of spacers supporting the current study with 55 isolates of *V. parahaemolyticus*. Present work has demonstrated that clinical isolates do not exhibit significant ITS1 length heterogeneity in repeated PCR analysis and thus the varying banding intensities offer further evidence to support the case for multiple amplicons. Additionally, pandemic O3:K6 isolates that were used in the current study show identical banding profiles to other clinical and environmental isolates. Based on these findings, ITS1 lengths (at least of the clinical isolates used in the study) correspond favourably to those of sequenced strains. These lengths take into account a subtraction of 112 bps corresponding to the primer positions near the 16S and on the 23S regions (Table 6.2). Based on these initial assumptions, it seems likely that the ITS1 Spacer Classes 0 (possessing no tRNA genes) and IA (possessing genes tRNA₁le and tRNA₁le') present in three and two copies respectively, in the sequenced strains are commonly found in *V. parahaemolyticus*. However the significance of having multiple copies of certain classes of operons remains unclear. Based on band intensities, it is probable that some environmental isolates may possess variations in operon copy number, a phenomenon suggested by others in *B. cereus* (Candelon *et al.*, 2004, *Clostridium difficile* (Sadeghifard *et al.*, 2006) and
*Acinetobacter* spp. (Maslunka, et al., 2006). The present data also suggest that Spacer Class E, possessing a single tRNA\textsuperscript{Glu} gene, was the most variable of regions supporting previous work showing that indels near the tRNA\textsuperscript{Glu} gene was responsible for ITS1 spacer variation (Gonzalez-Escalona *et al*., 2006). The presence of one, two or both bands (540 and 590 bps) in some of the isolates in the current work suggest that some isolates may possess two copies of this spacer, rather than just the one identified in previous studies (Makino *et al*., 2003, Gonzalez-Escalona *et al*., 2006a). The presence of these two bands may also correlate with the two *rrs* classes identified in *V. parahaemolyticus* (Moreno *et al*., 2002; Harth *et al*., 2007). Clearly, further work and sequence analysis would be required to verify any of these claims and establish the significance of these two bands and spacer classes to the virulence of *V. parahaemolyticus*.

This work has shown that multiple rRNA operons present in the *V. parahaemolyticus* genome can be exploited for the sub-typing of this organism using a simple PCR reaction. The primers used in the study identified additional ITS1 spacers in environmental isolates that previously had not been reported. The main ITS1 spacers (i.e. the more intense bands) consisting of multiple ITS1 regions offered a quick method to differentiate *V. parahaemolyticus* from other members of the aquatic environment but clinical isolates in general appeared indistinguishable from environmental strains with ITS1-PCR. Nonetheless, a previous study has shown that ITS spacers can be resolved directly from bacterial lysates without the need for further DNA purification (Gonzalez *et al*., 2003) thus favouring ITS-PCR for *V. parahaemolyticus*. The large numbers of genes for tRNA molecules present within the ITS1 spacer regions also suggest that a similar method may be employed to identify length polymorphisms in *V. parahaemolyticus* and is explored further in the next chapter.
Chapter 7

DNA PROFILING OF *VIBRIO PARAHAEOMOLYTICUS* ISOLATES USING tDNA-PCR TO GIVE INTERGENIC LENGTH POLYMORPHISMS (ILPs)

7.1 Introduction

The genes for tRNA can be found arranged in clusters distributed throughout the bacterial genome often within multiple tandem repeat units. The order and arrangement of these genes is highly conserved, whereas the intergenic spacer regions between them can vary considerably, being prone to the insertion of mobile genetic elements such as insertion sequences (IS), transposons, bacteriophages and genomic islands (Gurtler & Mayall, 2001). Length polymorphisms between adjacently primed tDNA sequences have been used to assess the degree of relatedness in a wide range of bacterial species and subspecies. The PCR amplification of tDNA intergenic length polymorphisms (tDNA-ILPs) can be achieved by employing consensus primers, or specifically designed primers that are annealed at low stringency. Welsh and McClelland developed a set of consensus tRNA gene primers designed to face outwards from the end of the tRNA genes that have been shown to amplify a tDNA-PCR fingerprint that is determined by the arrangement of tRNA genes on the bacterial genome (Welsh & McClelland, 1991a; McClelland et al., 1992). This technique has been successfully applied to distinguish at the species and subspecies level in a wide group of bacteria including *Renibacterium salmoninarum* (Alexander et al., 2001), *Streptococcus* (McClelland et al., 1992; Zschöck et al., 2005), *Staphylococcus* (Welsh & McClelland, 1992; Maes et al., 1997), *Acinetobacter* (Ehrenstein et al., 1996) and more recently in *Klebsiella pneumoniae* (Lopes et al., 2007).
Genome sequence information for *V. parahaemolyticus* has shown the organism to possess up to 126 tRNA genes distributed between the two chromosomes in comparison to 11 rRNA operons (Makino *et al*., 2003; Gonzalez-Escalona *et al*., 2005b). Thus, the successful application of consensus primers targeted to the tRNA regions of the *V. parahaemolyticus* genome has a greater banding pattern potential than 16S-23S ITS1-PCR (Chapter 6) to show intra-genomic variation, and hence provide a simple and rapid means of typing of this organism. The primers developed by Welsh and McClelland were based on 1) at least a perfect match in five bases between the 3' end of the primer and many tRNA genes and 2) extensive homology in the rest of the primer with a number of different tRNA genes from a wide variety of sources (Welsh & McClelland, 1991a). The recent use of these consensus primers with *Klebsiella* spp. revealed different tRNA arrangements and highlighted intraspecies heterogeneity (Lopes *et al*., 2007). Considering the ease of use and simplicity, this method could show high efficiency for the typing of *V. parahaemolyticus*.

This chapter describes the use of consensus primers developed by Welsh and McClelland and the development of a PCR method to assess the differentiation of *V. parahaemolyticus*. Thus the following were investigated:

i) molecular typing of *V. parahaemolyticus* based upon PCR amplification of tRNA spacer regions

ii) comparative analysis of different sets of consensus primers described by Welsh and McClelland (1991) with *V. parahaemolyticus*

iii) the usefulness of tRNA-PCR ILPs typing as a molecular typing tool in *V. parahaemolyticus*

iv) an evaluation of the ability to discriminate clinical and environmental (that may also be pathogenic) isolates of *V. parahaemolyticus* based on tDNA-PCR ILPs typing.
7.2 Materials and Methods

7.2.1 tDNA primer screening with *V. parahaemolyticus*

Primers T3A, T3B, T5A and T5B (Table 7.1) were initially screened in all 6 pair wise combinations on a panel of strains that included NCTC reference strain VP10884 and clinical and environmental isolates, in accordance with the published protocols (Welsh & McClelland 1991, 1992; Clementino *et al.*, 2001) and reaction conditions optimised for use with *V. parahaemolyticus* genomic DNA.

Table 7.1: Description of consensus tDNA primers used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3A</td>
<td>5'-GGGGGTTCGAA TTCCCGCCGGCCCCA-3'</td>
<td>Welsh &amp; McClelland, 1991a</td>
</tr>
<tr>
<td>T5A</td>
<td>5'-AGTCCGGTGCTCTAAACCAACTGAG-3'</td>
<td>McClelland <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>T5B</td>
<td>5'-AATGCTCTACCAACTGAATC-3'</td>
<td></td>
</tr>
<tr>
<td>T3B</td>
<td>5'-AGGTCGCGGGTTCGAATCC-3'</td>
<td></td>
</tr>
</tbody>
</table>

7.2.2 PCR amplification of tDNA intergenic spacer regions using consensus primers

The tDNA-PCR reactions were performed with DNA extracted from 56 isolates of *V. parahaemolyticus* and a further ten species that included other members of *Vibrionaceae*, *Listonella* spp., *Klebsiella* spp., and *E. coli*. Of the six primer sets, three were used to deduce fingerprinting profiles for all 69 isolates studied, viz. primer set 1 (T3A/T5A), primer set 3 (T3B/T5A) and primer set 5 (T3A/T3B); fingerprints were designated tD1, tD2 and tD3 respectively.

Reactions were performed using a 50 μl mixture containing 1 U of *Taq* polymerase and reaction buffer with 1.5 mM MgCl₂ (Roche), 20 pmol of each tRNA gene primer (Sigma), 0.2 mM deoxynucleoside triphosphates, and 20 ng of bacterial DNA. PCR amplification was facilitated in a DNA thermal cycler (Model: Primus 96, MWG Biotech). The reaction
mixture was incubated at 94°C for 2 min, and then subjected to 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min and a final cycle of 94°C for 30 s, 50°C for 30 s, and 72°C for 10 min. The amplification products were visualised after electrophoresis and banding profiles compared on 2% agarose gels. Banding profiles were also compared for reproducibility from two independent experiments.

7.2.3 Dendrogram analysis of tDNA-PCR

Dendrograms showing the relationships between isolates of *V. parahaemolyticus* were generated using BioNumerics Software with modules for Fingerprints and Cluster Analysis (version 5.1, Applied Maths, Ghent, Belgium). All banding patterns for the three consensus primer sets were used for the cluster analysis of 56 isolates of *V. parahaemolyticus*. The bands from each lane were subjected to curve calculations and analysis to exclude background noise from PCR reactions. The tracks were then normalised using two external reference positions that consisted of 50 bp molecular weight markers on either side of the gel and a common band that appeared in several lanes as an internal reference. Up to nine bands per lane, many of which were also common between isolates, were scored according to their presence or absence within the 100 to 800 bp range only. Dendrograms calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using both band matching coefficients (Dice) and curve matching correlation coefficients (Pearson product-moment correlation) were used as appropriate to obtain the most reliable clustering patterns. A dendrogram that combined the data for all three fingerprint sets was also constructed. BioNumerics position tolerance settings were 0.5% optimisation, a position tolerance of 5% of gel and ignoring faint bands.

7.2.4 Concordance between tDNA fingerprint types

The concordance between tD1, tD2 and tD3 fingerprint types were determined using BioNumerics by comparing the values from the similarity matrices of the typing methods
that were plotted on x and y axes. Each dot on a scatterplot represents corresponding similarity values for two isolates by different primer pairs given on the x and y axes and thus gives an indication of the degree of concordance between two techniques. The Pearson product-moment correlation coefficient was used to calculate the congruence between the three comparisons. The regression, r, was also calculated in BioNumerics.
7.3 Results

7.3.1 Consensus tDNA primer combinations

All six combinations of the four primers described by Welsh and McClelland were used on a test panel consisting of three clinical and three environmental isolates of *V. parahaemolyticus*. These primers were designed to amplify the intergenic spacer region from the 5' and 3' ends of tRNA genes and were selected based on the successful amplification of the most number of bands that were widely spread in fragment size (Fig 7.1). Primer pairs T3B/T5B and T3A/T5B showed a similar banding profile to T3B/T5A and T3A/T5A respectively, but fewer bands. Similarly, primers T5A/T5B showed a maximum of three bands amongst the six panel strains and therefore the remaining three primer pairs T3A/T5A, T3B/T5A and T3A/T3B were selected to investigate their ability to produce reproducible tDNA fingerprinting patterns and assess the ability to subtype clinical and environmental isolates of *V. parahaemolyticus*.

7.3.2 tDNA-PCR subtyping based on primers T3A and T5A (tD1)

The use of primers T3A/T5A (tD1) produced fingerprints that included four major bands of approximately 205, 355, 410 and 740 bp in most of the isolates of *V. parahaemolyticus*. Additional bands were present but appeared faint and were excluded from the analysis. Most of the differences in fingerprinting profiles were based on small but significant differences in the relative sizes of fragments that were amplified (Figs. 7.2 and Fig. 7.3).

Using UPGMA cluster analyses on *V. parahaemolyticus* is was possible to group the 56 isolates of *V. parahaemolyticus* into seven clusters at a 90% similarity value, labelled tD1-A to tD1-G (Fig. 7.3). Cluster tD1-A was the largest (25 isolates in fingerprint type tD1) but unable to differentiate three clinical UK isolates from the remaining 22 environmental isolates. Similarly, two Japanese clinical strains were grouped with 7 environmental isolates in cluster tD1-C. However, cluster tD1-D consisted entirely of 13 clinical isolates.
that included all 10 isolates from Norway, two from Japan and a UK clinical reference strain. Although these isolates were from three different locations, all 13 isolates were grouped into a single cluster that included isolates of diverse serotypes and Pulse Types (section 5.3.5). Cluster tD1-F contained five isolates of environmental origin and from the same geographical region. Isolates Vib8 and 6316 could not be grouped into any clusters; therefore, in this study they are the single representatives of two other groups tD1-E and tD1-G respectively (Fig. 7.3). As with Chapter 6, together with fingerprint types tD2 and tD3, the identity of isolate 6316 as *V. parahaemolyticus* remained questionable.

### 7.3.3 tDNA-PCR subtyping based on primers T3B and T5A (tD2)

Fingerprints produced by the primers T3B/T5A (tD2) showed a major band of approximately 370 bp that was present in all but one isolate (6316) of *V. parahaemolyticus* (Fig. 7.4). A further nine bands of less intensity ranging in size between 100 and 500 bp were also present in most of the isolates and could be used to distinguish *V. parahaemolyticus* into 8 types (Fig. 7.4: E). However, as with primers T3A/T5A, faint bands were excluded in the BioNumerics cluster analysis (Fig. 7.5).

Cluster analysis at a similarity value of 95% enabled the 56 isolates of *V. parahaemolyticus* to be grouped into 7 clusters (tD2-A to tD2-G). Cluster tD2-A was the largest accounting for 64% of the isolates but was unable to differentiate between a total of 36 clinical and environmental isolates at 95% similarity (Fig. 7.5). Group tD2-B was the only group to consist of clinical isolates, both of which were from Norway. Clusters tD2-C and tD2-F both consisted of a mixture of clinical and environmental isolates, whereas tD2-D and tD2-E contained environmental isolates only.
7.3.4 tDNA-PCR subtyping based on primers T3A and T3B (tD3)

Primers T3A/T3B (tD3) amplified a maximum of nine visible amplicons with three major bands of approximately 240, 270 and 500 bp that were present in all but one isolate of *V. parahaemolyticus* (6316). A distinct feature of tDNA-ILPs with these primers was that the sizes of all bands fell within a relatively short range of 240-550 bp (Fig. 7.6).

At a 95% similarity value, a UPGMA cluster analysis grouped the 56 isolates into 9 clusters (tD3-A to tD3-I). Cluster tD3-B appeared the largest consisting of 26 clinical and environmental isolates (Fig. 7.7). Two other clusters (tD3-A and tD3-C) also consisted of mixed clinical and environmental isolates. Group tD3-D contained three clinical strains and NCTC UK reference strain, VP10884 (Fig. 7.7). In addition, two clinical strains (219 and E155855) were clustered with an otherwise predominantly environmental group of isolates in clusters B and C respectively. Three Norwegian clinical isolates that were previously grouped with other clinical isolates (based on fingerprint types tD1 and tD2) from the same geographical region fell into a distinct cluster (tD3-D) of its own (Fig. 7.7). Furthermore, using primers T3A and T3B isolates VPSCI, 2053 6316 and 1021/5 represented unique groups of their own.

7.3.5 Comparative analysis of tDNA fingerprinting with different primers

Using a curve based correlation coefficient to generate dendrograms for all three primer sets, it was possible to group isolates at relatively high similarity values of >90%. With dendrogram analysis the 56 isolates of *V. parahaemolyticus* were grouped into seven, seven and nine clusters for the three fingerprint types, tD1, tD2 and tD3 respectively, as described above. With the exclusion of isolate 6316 that was distinguished into its own type with all three primer sets, the remaining 55 isolates showed similarities greater than 66%, 93% and 83% in each of the fingerprint types tD1, tD2 and tD3 respectively (Figs. 7.3, 7.5 & 7.7). Of the groups obtained, there appeared some concordance in groupings
amongst the three fingerprint types. For example, four Spanish isolates (1022/10, 1023/1, 1023/3 and 1103/9) grouped in the first cluster of tD1 and tD2 were also sub-grouped in tD3-C but a further isolate (1021/5) was not identified as concordant. Likewise, whereas two isolates (VP349 & 2053) from tD1-B were found together, they were identified in separate groups in tD2 and tD3. Similar qualitative measures of concordance could be identified throughout the three fingerprint types.

A comparison of the similarity matrices obtained from the three primer pairs was performed (Fig. 7.8). Three scatterplots representing all three comparisons demonstrated data skew towards high percentage similarity values whereas plots at the lower percentages were representative of distance relatedness of isolate 6316, the identity of which remained uncertain from typing experiments. A measure of concordance calculated with the Pearson product-moment correlation coefficient yielded congruence values of 69.9, 47.2 and 74.6 percent for comparisons tD1/tD2, tD1/tD3 and tD2/tD3 respectively (Fig. 7.8). Linear regression models showed good data fit of 75, 66 and 82% for the respective comparisons (Fig. 7.8) Therefore, fingerprint types tD2 and tD3 were identified most concordant and tD1 and tD3 as the least concordant of the three comparisons.

7.3.6 Combined dendrogram analysis of tDNA-PCR

Fingerprints tD1, tD2 and tD3 were combined as a composite data set (tD-combined) to assess whether better discrimination between V. parahaemolyticus isolates could be achieved (Fig. 7.9). At 95% similarity, isolates were grouped into 12 clusters (tD-combined A to L) of which five isolates (1024/5, VP939, 2436, 2053 & 6316) formed single members of their own clusters. With the identity of isolate 6316 questioned, the remaining 55 isolates appeared >84.5% similar in combined dendrogram analysis. Cluster tD-combined -C grouped three clinical strains with environmental isolates from Southampton whereas Clusters D, E, J and K consisted entirely of environmental isolates.
Group F clustered 12 clinical strains with NCTC reference strain VP10884. The composite tD-combined data did not discriminate more than single tDNA primer pairs alone. tD1 formed a larger number of groups at 95% similarity but groupings appeared to correspond largely to the isolates from the same gels. The combined data is evaluated for concordance with other typing systems in Chapter 10.

7.3.7 Reproducibility of tDNA-PCR

A side-by-side comparison of six isolates of *V. parahaemolyticus* was performed to assess the reproducibility of the tDNA-PCR technique (Fig. 7.10) with all three sets of primers. A total of 18 comparisons of two repeats were analysed. Isolates from tDNA-PCR described in the reproducibility test were obtained from Figures 7.1 (20 pmols of primers), 7.2, 7.4 and 7.6.

Fingerprints appeared largely reproducible with regards to presence or absence of bright bands, but faint bands showed more variability (Fig. 7.10). For example, in some of the repeats, faint bands could not always be ascertained, appeared absent or present in the repeat. Additionally, in some tests the brighter bands did not appear consistent between the two repeats (tD1: 1021/5; tD3: 1021/5 & 2053). However, of the 18 comparisons, the main amplicons (4 in tD1, 1 in tD2 and 3 in tD3) could be identified in all but one comparison (Primer set tD1, isolate VP10884) (Fig. 7.10).

7.3.8 tDNA-ILPs in other species

Distinct fingerprint profiles were obtained for isolates of *V. anguillarum*, *V. mimicus*, *V. fluvialis*, *V. cholerae*, *V. vulnificus*, *V. alginolyticus* and *L. damsela* with PCR conditions optimised for *V. parahaemolyticus* in all fingerprint types (Figs. 7.2, 7.4 & 7.6). In all three fingerprint types isolate 6316, which had previously shown a unique fingerprint pattern, matched that of *V. mimicus* (Figs. 7.2, 7.4 & 7.6). Additionally, the profile of isolate Vib4
an unspeciated *Vibrio* sp., matched that of *L. damsela* (LD365). Furthermore, common amplicons were present in several *Vibrio* spp. For example, with primers T3B and T5A, *V. cholerae* and *V. parahaemolyticus* shared two common sized fragments of approximately 200 and 470 bp (Fig 7.3). Similarly, *V. mimicus* and *V. vulnificus* shared common bands with *V. parahaemolyticus*. Dendrogram analysis revealed the close tDNA-ILP similarities of *V. alginolyticus* (2341) and *V. vulnificus* (KIDBVV) with that of *V. parahaemolyticus* (Fig. 7.11). The data showed a high degree of consistency of the tDNA-PCR technique in differentiating *V. parahaemolyticus* at the species level.
Figure 7.1: tDNA-PCR fingerprinting profiles with all 6 combinations of tDNA primers using a panel strain of 3 clinical and 3 environmental isolates. Lanes corresponding to the following isolates: 1-5: 1021/5; 6-10: 2053; 11-15: Vib1; 16-20: E155855; 21-25: VP350; 26-30: NCTC reference strain VP10884. In each set of 5 lanes, the first two lanes correspond to 10 pmol of primers, and the second two lanes correspond to 20 pmol of primers. Each lane alternates with the first and second primer set in Gels 1, 2 and 3 above. The fifth lane for each isolate is DNA only. Negative controls are marked with - and M corresponds to 50 bp molecular size markers: selected sizes are indicated on the right of each gel.
Figure 7.2: tDNA-PCR fingerprints of 56 isolates of *V. parahaemolyticus* and related species of clinical and environmental origin using primers T3A and T5A. Lane marked (-) is no DNA control and each lane is marked with the corresponding isolate at the top. NCIMB and NCTC reference strains for *V. parahaemolyticus* are 1902 and VP10884 respectively. Strains 11327 and 8042 are NCTC reference strains for *V. vulnificus* and *V. cholerae* respectively. In each gel, 50 bp ladders were used, sizes of selected markers are indicated by arrows on the right.
Figure 7.3: Dendrogram based on UPGMA clustering of normalised tDNA-ILPs of *V. parahaemolyticus* obtained with primers T3A and T5A. At least 4 bands were present in all isolates (except 6316), varying in size and intensity. Clusters tD1-A to tD1-F are based on a 90% similarity score indicated by the dotted line. Scale above banding profiles indicates approximate sizes of amplicons.
Figure 7.4: tDNA-PCR fingerprints of 56 isolates of *V. parahaemolyticus* and related species of clinical and environmental origin using primers T3B and T5A. Lane marked (-) is no DNA control and each lane is marked with the corresponding isolate at the top. NCIMB and NCTC reference strains for *V. parahaemolyticus* are 1902 and VP10884 respectively. Strains 11327 and 8042 are NCTC reference strains for *V. vulnificus* and *V. cholerae* respectively. In each gel 50 bp ladders were used, sizes of selected markers are indicated by arrows on the right. Insert (E) shows the eight tD2 banding types identified using primers T3B/T5A.
Figure 7.5: Dendrogram based on UPGMA clustering of normalised tDNA-ILPs of *V. parahaemolyticus* obtained with primers T3B and T5A. Up to eight bands were present in most isolates (except 6316), varying in size and intensity. Clusters A to G are based on a 95% similarity score indicated by the dotted line. Scale above banding profiles indicates approximate sizes of amplicons.
Figure 7.6: tDNA-PCR fingerprints of 56 isolates of *V. parahaemolyticus* and related species of clinical and environmental origin using primers T3A and T3B. Lane marked (-) is no DNA control and each lane is marked with the corresponding isolate at the top. NCIMB and NCTC reference strains for *V. parahaemolyticus* are 1902 and VP10884 respectively. Strain 11327 and 8042 are NCTC reference strains for *V. vulnificus* and *V. cholerae* respectively. In each gel 50 bp ladders were used, sizes of selected markers are indicated by arrows on the right.
Figure 7.7: Dendrogram based on UPGMA clustering of normalised tDNA-ILPs of *V. parahaemolyticus* obtained with primers T3A and T3B. Up to 10 bands were present in all isolates (except 6316), varying in size and intensity. Clusters tD3-A to tD3-I are based on a 95% similarity score indicated by the dotted line. Scale above banding profiles indicates approximate sizes of amplicons.
Figure 7.8: Similarity plots of tDNA-PCR fingerprint types with three primer pairs. Congruence of fingerprint types tD1/tD2, tD1/tD3 and tD2/tD3 were 69.9%, 47.2% and 74.6% respectively. The linear regression correlation, r, is also calculated and expressed as a percentage fit (BioNumerics, Applied Maths).
Figure 7.9: Combined dendrogram of composite tD1, tD2 and tD3 fingerprint data. 12 clusters tD-combined -A to L are based on a 95% similarity score indicated by the dotted line.
Figure 7.10: Reproducibility of tDNA-PCR fingerprints from six isolates in repeat tests 1 and 2 with all three primer pairs. Arrows indicate common amplicon of approximately 355, 370 and 270 base pairs in fingerprint types tD1, tD2 and tD3 respectively and indicate relative migration of bands on each gel.
Figure 7.11: Dendrogram showing relatedness based on UPGMA clustering of tDNA-ILPs of *V. parahaemolyticus* and 8 related species. tDNA consensus primers T3A and T5A were used and data analysed with a curve based Pearson product-moment correlation coefficient.
7.4 Discussion

To the author’s knowledge, this chapter is the first study that assesses tDNA-ILP typing for *V. parahaemolyticus* by comparing and evaluating the performance of three pairs of tDNA primers. The use of these primers provided a simple and largely reproducible PCR based approach to subtype clinical and environmental isolates of *V. parahaemolyticus*. This created divisions between isolates that had previously been shown to be biochemically and serologically indistinguishable and where clinical and environmental isolates could not be grouped by other means (Chapters 4 and 6). The method developed was applied to a total of 36 environmental and 20 clinical isolates sourced from the UK, Spain, Norway and Japan as well as isolates from other species. Based on the present study, the identity of one clinical *V. parahaemolyticus* isolate, 6316, remained questionable.

The tRNA genes occur in multiple copies dispersed throughout the bacterial genome in most species. The shared sequence motifs of tRNA genes mean that the use of primers that contain consensus tRNA sequences in the PCR are likely to result in a number of characteristic PCR products (Welsh & McClelland, 1991a). In the current study, the use of consensus primers enabled the subgrouping of *V. parahaemolyticus* isolates into distinct clusters based on a similarly score of greater than 90%. These groups, although not identical, fared well in concordance studies with correlation greater than 66%.

7.4.1 Fingerprinting based on tD1, tD2 and tD3

Up to four bright bands and a further five faint bands were common in most isolates with tD1 and these are representative of the multiple copies of tRNA genes found within clusters, which in turn are arranged within tandem repeat units. These bands can be generated from both within and between these multiple repeat regions (Honeycutt *et al.*, 1995). Upon closer inspection, it can also be seen that variation lies mainly in the relative sizes of the fragments and intensity of some of the bands rather than in the presence or
absence of bands, except for one clinical isolate, 222. Here, two faint bands were absent altogether, suggesting the absence or sequence variation of primer binding sites present in all the other strains. The absence of such bands also highlights a major drawback in the use of consensus tDNA primers in producing a robust test. Whereas mismatches between primer and target sequence can give rise to variation evident by PCR, the same mismatches compromise the stability and hence the reproducibility of faint bands evident in some of the repeat tests. However, a trade-off between reasonably reproducible results and ease of use formed the basis of the present study.

With the large degree of similarities in banding profiles (similarity value of 75% accounting for 89% of all isolates), it was decided to cluster tD1 groups at 90% similarity values, resulting in large clusters and only seven divisions but interestingly formed an entirely new clinical division that had previously not been clustered using PFGE. Of this group, four isolates were of the pandemic serotypes, O3:K6 (isolates 220, 222 and 223) and O1:KUT (isolate 221). Isolates of these serovars have repeatedly shown a high degree of clonality in previous studies (Okuda et al., 1997b; Chowdhury et al., 2000b; Matsumoto et al., 2000; Laohapretrthisan et al., 2003). Based on tDNA-PCR it appears that these clonally related groups fell within a group of 13 clinical isolates of diverse serovars forming a unique cluster. However, as all 13 isolates were from the same gel (Fig. 7.2: Gel B), careful interpretation is necessary to determine whether this grouping could be attributed to close banding similarities or due to the inability of BioNumerics to differentiate isolates on the same gel, especially at such high similarity values. A further criticism of the method developed is the use of an internal marker based on common bands in a gel in the BioNumerics normalisation process. Additional external markers (i.e. molecular size markers within the gel) would have been preferable, but was omitted from the experimental design as the author was unaware at the time that data would be analysed in BioNumerics. These errors in experimental design need to be addressed in future studies.
to validate such groupings. Nonetheless, a further two clonally related O3:K6 isolates (5421, 2053, Table 4.3) from cluster tD1-C showed a similar degree of relatedness to each other and were on the same gel, but failed to be clustered in tD1-D. Group specific PCR (GS-PCR) markers for the post 1995 clonal pandemic strains (Bhuiyan et al., 2002), have been shown to be positive for eight out of the 13 isolates including the two clinical isolates of tD1-B (Pers comm: S. Wagley).

Environmental isolates of tD1-F all originating from nearby oyster (Crassostrea gigas) samples showed similar profiles. However, two isolates VP491 and VP939, isolated from the same region but different sources: scallop (Pecten maximus) depuration and mussel (Mytilus edulis) samples respectively, were mapped differently in the dendrogram. The observation that isolate VP491 clustered with environmental isolates from Spain is particularly interesting since environmental strains of V. parahaemolyticus have been known to exist as genetically heterogeneous populations in comparison to clinical isolates (Chapter 5; Wong et al., 1999a; Martinez-Urtaza et al., 2004). Despite isolates being obtained from different geographical regions, intergenic regions, at least with primers T3A and T5A, between tRNA gene clusters appeared the same.

Whereas dendrogram analysis seemed to have differentiated tD1 isolates based on small differences in fragment sizes and banding intensity of four amplicons, primers for tD2 produced eight unique fingerprint patterns but only a single intense band. As mentioned earlier, faint bands did not always appear consistent in repeat comparisons of isolates in tD2 and therefore it is presently not clear whether such banding patterns could be used as reference types. It is also worthy of note that only two repeats were compared to assess the reproducibility of tDNA-PCR. Clearly, a further series of repeats, together with more accurate band sizing techniques would be necessary to substantiate the eight tD2 types described. Additionally, by combining three or more repeats in BioNumerics to produce an
averaged curve based banding pattern, it may have been possible to reduce inconsistencies of band intensities and to overcome this drawback of the use of consensus primers.

With large clusters based on high percentage similarities, empirical assessment of concordance in clusters between tD1 and tD2 became difficult. It became apparent that although some isolates from the groups of two fingerprint types were concordant (as expected with such large clusters), others were not. A scatterplot comparing the values of percentage similarity between two fingerprint types demonstrated the skew of data towards high matrix similarity figures. A congruence value approaching 70% between tD1 and tD2 (regression = 75.0%) suggested a high degree of concordance between tD1 and tD2 fingerprint types; however, it is not known to what extent this could be attributed to isolates being placed on the same gel in each of the fingerprint types and further emphasises the need for random distribution of tDNA-PCR products between agarose gels.

It is of particular interest that the Norwegian isolates that had previously been grouped together, largely in one main group (clusters D and A for fingerprint types tD1 and tD2 respectively), was found split in two distinct groups (clusters B and D) suggesting some discrimination of clonally related isolates in tD3. Concordance studies of tD3 with fingerprint types tD1 and tD2 provided some confirmatory results. Whereas tD1 and tD3 appeared the least congruent, fingerprint types tD2 and tD3 exhibited a greater degree of concordance. These results may reflect that greater variation in tD1 and least variation in tD2 (55 V. parahaemolyticus isolates showed > 66% similar in tD1, >92% similarity in tD2 and >88% similarity in tD3) could be partially responsible for the congruence values in the three fingerprint types. A combined dendrogram of composite data, as expected, produced a greater number of clusters at 95% similarity than tD2 or tD3, but failed to give more discrimination than tD1. Interestingly, in congruence tests (Chapter 10, Fig. 10.8) it appears that the composite data, tD-combined, is more congruent with tD1 than tD2 or
tD3 and thereby confirming primers T3A and T5A for better discrimination of *V. parahaemolyticus*.

### 7.4.2 Consensus and specific tDNA primers

The primers designed by Welsh and McClelland were based on consensus tRNA sequences from a number of bacterial species (Welsh & McClelland, 1991a, 1992). Preliminary work investigated the potential development and use of specific *V. parahaemolyticus* tRNA primers. Based on availability of a single genome sequence, it was found that tDNA sequences between tRNAs for different amino acids varied considerably. Copies of the same tRNA genes were not found clustered adequately to yield an appropriate number and size of intragenomic length polymorphisms. BLAST analysis of consensus primer sequences designed by Welsh and McClelland identified significant homologies to the tRNA genes of *V. parahaemolyticus*. Of the three primers (T3A, T3B and T5A) that were investigated by BLAST searches, three, six and ten tDNA sites were found to be homologous to the primers T3A, T3B and T5A respectively, with 12 to 17 bp homology and only a single bp mismatch. It is likely that those were the priming sites and it is therefore unlikely that the use of specific tDNA primers would have been able to further differentiate *V. parahaemolyticus*.

### 7.4.3 General considerations from tDNA-PCR typing

This chapter has demonstrated that tDNA-PCR can serve as a reasonably discriminatory epidemiological typing method for *V. parahaemolyticus* that is easy to execute and relatively cost effective. As the method is less time consuming than others such as ribotyping, tDNA-PCR could be implemented to screen a larger number of environmental samples. However, several important considerations need to be made. Firstly, as with other typing systems, the stability of the tDNA-PCR needs to be assessed. The stability of a typing system is defined as the ability to express constant markers over time and
generations, and the method should be stable for at least the time frame of the investigation (Struelens, 1998). Length polymorphisms of tRNA spacer regions may not necessarily be coding regions and hence their stability could be questioned (Burr et al., 1998). Other than identifying isolates in distinct clusters in independent fingerprinting experiments, this study has not fully assessed the stability of the tDNA-PCR technique over a period of time, and this should be explored if tDNA-PCR is to be implemented for routine monitoring.

Reproducibility is a second factor for consideration. In fingerprint types tD1, tD2 and tD3, all but one isolate (6316) was clustered together at similarity coefficients of 67%, 92% and 89% respectively. The presence of intense bands common to most isolates in repeat tests demonstrated a fairly good degree of reproducibility of this technique. An inter-laboratory study that incorporated capillary electrophoresis in the identification of Streptococcus spp also concluded reproducibility in tDNA-PCR (Baele et al., 2001). These findings are consistent with those of several other workers (Baele et al., 2000; De Gheldre et al., 2001; Clementino et al, 2001, Lopes et al., 2007).

In addition to stability and reproducibility, the discriminatory powers of tRNA primer sets and typeability are of major importance. Clearly, the three sets of primers grouped isolates at different similarity coefficients and gave different groupings. Primer set tD1 was most discriminatory and tD2 least discriminatory based on a few amplification products. Ease of data interpretation is another factor that is applicable with tDNA-PCR. Almost all typing experiments require the use of data analysis software. The large numbers of groups obtained are the result of sophisticated data manipulation, normalizations and curve calculations. Clearly, without the aid of such fingerprint analysis software, such groupings would not have been possible.
7.4.4 Typing of *V. parahaemolyticus* based on tDNA spacer regions

As with 16S - 23S ITS-PCR (Chapter 6), tDNA-PCR discriminates between isolates by measuring molecular variation within defined regions, i.e. the tRNA gene spacer regions both between and within tRNA gene clusters. Up to nine amplicons were generated using the various primer pairs, and in each case, variation was found mainly in the size and intensity of the bands produced, although the number of amplicons was the same in each fingerprint type in most isolates. This could suggest that numbers of tRNA genes remain the same in most isolates. A total of 126 tRNA genes in *V. parahaemolyticus* (in strain RIMD) is relatively high compared to that found in *E. coli* K12, *E. coli* O157 and *S. typhimurium* (86, 99 and 85 for the three strains respectively) and this may correlate with the comparatively high ribosomal operon number, reflecting *V. parahaemolyticus* fast growth properties (Makino *et al.*, 2003; Withers *et al.*, 2006). However, from tDNA-PCR fingerprint data alone, it was not possible to deduce possible variations in copy numbers of tRNA genes amongst *V. parahaemolyticus*. As both methods of spacer regions analysis (tDNA-PCR and ITS-PCR) were looking at specific genomic region, it was logical to examine the isolates further, through a method which gives access to the entire genome. Although PFGE studies aimed to establish this, a lack of comprehensive typing data resulted in the development of a sensitive RAPD method described in Chapter 8.
Chapter 8

RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) METHOD FOR DIFFERENTIATING VIBRIO PARAHAEOMOLYTICUS

8.1 Introduction

Randomly amplified polymorphic DNA (RAPD) is a PCR based technique where short primers (usually 10-mers) of arbitrary sequence are used to generate amplification products characteristic of the template DNA (Williams et al., 1990). The method is unique in that it requires no previous knowledge of the target genome sequence, requires only small quantities of template DNA, can be analysed with standard electrophoresis methods and be used for any organism. The simplicity of this arbitrarily primed PCR method (also known as AP-PCR) has enabled this technique to be adopted in a wide variety of applications such as determining parent-sibling connections (Hadrys et al., 1993), identification of genetic alterations in tumours (Dil-Afroz et al., 1998) and the detection of UV induced genotoxicity (Atienzar et al., 2000). In bacteriology, RAPD analysis has been successfully used to differentiate between strains of a wide variety of species including Renibacterium salmoninarum (Grayson et al., 1999, 2000), Burkholderia pseudomallei (Leelayuwan et al., 2000) and Vibrio vulnificus (Ryang et al., 1997). More recently, RAPD was used to differentiate clonally related V. cholerae that could not be discriminated by ribotyping or PFGE (Pourshafie et al., 2007).

Wong and colleagues (1999c) described the use of RAPD to establish relationships among 308 clinical isolates of V. parahaemolyticus from food poisoning outbreaks in Taiwan. A total of 41 polymorphic RAPD patterns were identified based on the presence of up to 10 bands that were grouped into 16 types (Wong et al., 1999c). Earlier work examined the genotypes of urease positive clinical strains of V. parahaemolyticus (Okuda et al., 1997a),
demonstrating that RAPD was able to differentiate between O4:K12 serotypes and non-O4:K12 serotypes in a sample of eight clinical isolates. In a further report, the clonal relationship of O3:K6 isolates from Calcutta and Japan was demonstrated with RAPD (Okuda et al., 1997b). Both of the studies by Okuda and colleagues used single primers P1 and P2 and, as expected, clonally derived isolates yielded similar comparative results. However, both single primers produced a mere five major amplicons and several faint bands that were hardly discernable from background PCR noise (Okuda et al., 1997a, 1997b). These primers, together with primers P3, P4, P5, P6 (Amersham Biosciences) were investigated by Maluping et al., (2005) who were only able to show a maximum of between 2 to 4 bands with primers P4 and P6 in environmentally acquired isolates and thus it was difficult to obtain sensitive discrimination of data. To date, there has been no attempt to assess the ability of a RAPD method to differentiate pathogenic isolates of *V. parahaemolyticus* from environmentally acquired samples.

Previous work that employed consensus ITS and tRNA gene primers showed up to eight distinct amplicons (Chapters 6 and 7 respectively) and enabled the grouping of isolates. However to further assess relatedness between isolates and to allow a greater probability for a possible discrimination in relationships, it was necessary to produce a more complex banding pattern. This chapter describes the use of combined RAPD primers that have been used in both bacteria (*Mycoplasma* spp., and *E.coli*), and plants (*Brassica napus*, and *Hordeum* spp.). Combined primers have been shown to demonstrate greater polymorphisms in banding than single primers alone (Hu et al., 1995; Reddy & Soliman, 1997; Charlton et al., 1999; Hopkins & Hilton, 2001).

The following are investigated in this chapter:

i) screening of commercially available RAPD primer pairs for complex and reliable banding patterns
ii) analysis of RAPD fingerprinting in *V. parahaemolyticus* based on two different primer sets for comparison and reliability of data produced.

iii) an evaluation of the ability to discriminate clinical and environmental isolates of *V. parahaemolyticus* based on RAPD fingerprinting.
8.2. Materials and Methods

8.2.1 RAPD - PCR of *V. parahaemolyticus*

RAPD analysis was performed on 56 isolates of *V. parahaemolyticus* using RAPD Analysis Beads (Amersham) containing 6 distinct random 10-mer primers as shown in Table 8.1. Each bead, when resuspended in a 25 µl volume contained 1 µl AmpliTaq DNA polymerase and Stoffel fragment, 0.4 mM of each dNTP, 2.5 µg BSA and buffer [3 mM MgCl$_2$, 30 mM KCl and 10 mM Tris, (pH 8.3)]. To each reaction bead 25 pmol (5 µl) of the appropriate primer and DNA template of appropriate volume equivalent to 10 ng, were added to generate optimum banding patterns upon agarose gel electrophoresis. The PCR reaction conditions were as follows: 1 cycle at 95°C for 5 min, followed by 45 cycles at 95°C for 1 min, 36°C for 1 min and 72°C for 2 min. All RAPD products were analysed by electrophoresis of 15 µl of each reaction mixture in 2% agarose gels as described in section 3.6.

<table>
<thead>
<tr>
<th>RAPD Primers</th>
<th>Primer Sequence</th>
<th>%GC</th>
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<tbody>
<tr>
<td>P1</td>
<td>5'-d[GGTGCGGGAA]-3'</td>
<td>70</td>
</tr>
<tr>
<td>P2</td>
<td>5'-d[GTTTCGCTCC]-3'</td>
<td>60</td>
</tr>
<tr>
<td>P3</td>
<td>5'-d[GTAGACCGGT]-3'</td>
<td>60</td>
</tr>
<tr>
<td>P4</td>
<td>5'-d[AAGAGCCCGT]-3'</td>
<td>60</td>
</tr>
<tr>
<td>P5</td>
<td>5'-d[AACGCCAACC]-3'</td>
<td>60</td>
</tr>
<tr>
<td>P6</td>
<td>5'-d[CCCGTCAGCA]-3'</td>
<td>70</td>
</tr>
</tbody>
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8.2.2 Screening of RAPD primers P1 to P6, singly and in pairs

Genomic DNA (section 3.3) from a panel of six isolates of *V. parahaemolyticus* was screened with single primer, and in all possible paired combinations (15 reactions) with primers P1 to P6. In order to determine the best discriminating banding patterns. Subsequently, primer pair P2 and P3 (designated primer set 1, PS1) was used on a larger panel of 18 isolates, before they were applied to all 56 isolates used in the study. Primer pair P1 and P5 (designated PS2) were also used as a duplicate set of experimental results to assess the reliability of RAPD clustering used in this method.
8.2.3 Dendrogram analysis with BioNumerics

Dendrograms showing relationships amongst clinical and environmental isolates of \textit{V. parahaemolyticus} were generated using BioNumerics Software with modules for Fingerprints and Cluster Analysis (version 5.1, Applied Maths, Belgium) as explained in section 6.2.3. All banding patterns for PS1 and PS2 were used for the cluster analysis of 56 isolates of \textit{V. parahaemolyticus} and to make comparisons between the two sets of data. The bands from each lane were subjected to curve calculations and analysis to exclude background noise from PCR reactions. The tracks were then normalised using two external reference positions that consisted of 100 bp molecular weight markers on either side of the gel, and a common band that appeared in several lanes as an internal reference. Up to 13 bands per lane, many of which were also common between isolates, were scored according to their presence or absence within the 100 - 2072 bp range only. Dendrograms were calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using a Pearson product-moment correlation coefficient. BioNumerics position tolerance settings were 1% optimisation, a position tolerance of 1% of gel and faint bands were ignored. Clusters were based on 90 \% similarity value in both fingerprint types, PS1 and PS2.

8.2.4 Concordance between RAPD - PS1 and PS2 typing

The degree of concordance between RAPD-PS1 and RAPD-PS2 was determined using BioNumerics software with modules for Fingerprints and Cluster Analysis (version 5.1, Applied Maths, Belgium). Values from the similarity matrices of the PS1 and PS2 typing methods were plotted by BioNumerics on an \(x\)-and \(y\)- graph. Each dot represents corresponding similarity values for two isolates by the typing methods given on the \(x\) and \(y\) axes and thereby gives an indication of concordance between PS1 and PS2 typing. The congruence between PS1 and PS2 was calculated using the Pearson product-moment correlation coefficient in BioNumerics.
8.3 Results

8.3.1 Single primer (P2) screening of *V. parahaemolyticus*

Two environmental isolates, VP350 and VPSCI, were screened with primer P2 that had previously been shown to amplify at least five prominent bands in clinical strains of *V. parahaemolyticus* (Okuda et al., 1997b). Between one and four bands were observed; these varied considerably based on DNA template concentration and primer concentration used (Fig. 8.1). Clearly, the use of a single primer failed to give the complexity of banding patterns sought.

8.3.2 Two-primer screening of RAPD reactions

Between four and 10 bands were observed when two primers were combined (Fig. 8.2). Out of the 15 different primer combinations, primers P3/P6, P3/P5, and P4/P4 showed the lowest number of bands, whilst most exhibited 5 – 10 amplicons. A greater number of bands were exhibited with primers P2/P3 whereas primers P1/P5 showed higher intensities of the two main amplicons generated. Based on these findings, primer combinations P2/P3 (designated PS1) and P1/P5 (designated PS2) were used to produce fingerprinting patterns of a panel strain of 18 clinical and environmental isolates of *V. parahaemolyticus* (Fig 8.3).

8.3.3 RAPD typing of *V. parahaemolyticus* and related species with PS1

A total of 56 isolates of *V. parahaemolyticus*, plus 14 isolates of other *Vibrio* spp., and nine non-vibrio species were typed using PS1 (Figs 8.4 to 8.8). There was 100% typeability, where all 79 isolates showed comparable banding profiles that largely fell within the range of the 100 bp size markers from 100 - 2072 bp (Fig 8.3). A comparison of RAPD-PCR reproducibility in a selection of isolates is described in section 8.3.5. Approximately nine to 20 bands of varying intensity and size were observed with *V. parahaemolyticus* isolates with at least nine common amplicons of various sizes. Distinct bands of around 180, 200, 280, 370, 590, 610, 860, 1000, and 1350 bp as well as several higher molecular weight
bands were amplified (Fig. 8.3). Significant differences in banding patterns in other *Vibrio* spp. were also identified. Six isolates of *V. cholerae* showed distinct banding patterns (Fig 8.7). Likewise, *V. anguillarum, V. alginolyticus, V. mimicus, V. fluvialis, L. damsela* and non-vibrios could also be differentiated by unique banding profiles (Figs. 8.5 to 8.8).

A common band (Band X) of approximately 910 bp was present in 10 out of the 20 clinical isolates studied. Other than the possibility of this band present in isolate 1103/9 (Figs. 8.5 & 8.9), no other environmental isolate seemed to possess this band. However, unlike the clinical isolates, Band X appeared very faint in 1103/9. A similar sized amplicon was also identified amongst some members of RAPD Types 22 and 23, but appeared characteristically smaller (Fig. 8.6) or faint (Fig. 8.8). The 10 clinical isolates were of the O3:K6, O3:KUT or O5: K68 serovars accounting for seven, two, and one isolate respectively (Fig. 8.9). Both O3:K6 isolates from the UK (E155855 & E168143) have been shown to be related to the pandemic clones (Wagley et al., 2008).

At a 90% similarity value based on dendrogram analysis, *V. parahaemolyticus* isolates were differentiated into 30 RAPD types (Fig. 8.10) with clinical isolates in RAPD types (RT) 10, 11, 12, 15, 21, 24, 26 and 27. Of these, four types only had single members (219, 224, 2436 and 6316) with the identity of isolate 6316 questioned from earlier chapters. An environmental isolate VP416, isolated from the common oyster, was clustered closely with a Japanese clinical isolate 6134. Clinical isolates from UK, Japan and Norway were clustered into distinct groups.

Of the 20 environmental types, 13 isolates fell into their own types and could not be grouped with other isolates at a 90% similarity level. However, PS1 clustered environmental isolates VP1 to VP4 together and VP6 to VP9 together into two groups (RT 20 and 16 respectively). Similarly four isolates, Vib1- Vib3 and Vib5 (RT 23) were found
to be more closely related than Vib6 - Vib10 (RT 22) from the Southampton environmental isolates (Fig 8.10). As with the environmental isolates from Spain, *V. parahaemolyticus* isolated from environmental samples in the UK fell generally into a heterogenic population.

### 8.3.4 RAPD fingerprinting of *V. parahaemolyticus* based on PS2

All 79 isolates that included members of other *Vibrio* spp and non-vibrios were also typed using a second set of primers P1 and P5 (PS). To ease analysis and comparison of data, only *V. parahaemolyticus* isolates are shown (Fig. 8.11). As with PS1, there was 100% typeability with approximately nine to 18 bands of varying intensity and size observed for *V. parahaemolyticus*. Most isolates showed at least six common amplicons of various sizes with bands predominantly around 130, 250, 310, 590, 730 and 1130 bp (Fig. 8.11).

At a 90% similarity value based on UPGMA dendrogram analysis, *V. parahaemolyticus* isolates were differentiated into 24 RAPD types (Fig. 8.12) with clinical isolates present in seven RAPD types; 02, 08, 15, 16, 18, 22 and 23. Of these, only one isolate (2436) constituted its own group (RT 16). RAPD Type 15 clustered two UK O3:K6 isolates with two Japanese O3:K6 isolates, whereas RT 22 and 23 grouped all the Norwegian isolates together. As with the previous primer set, the banding pattern of an environmental isolate, VP416, closely resembled Japanese clinical isolate, 6134. Furthermore, a common band Y of approximately 610 bp was present in 11 out of the 20 clinical isolates studied (Fig. 8.10). This band seemed to be present in some environmental isolates as well but as with Band X, appeared distinctly weaker. The 11 clinical isolates included those 10 previously described with PS1 that were of O3:K6, O3:KUT or O5:K68 serovars, with the addition of isolate 221 of the O1:KUT serotype (Fig. 8.12).
Of the 15 environmental types, six isolates were sole representatives of their own types and could not be grouped with other isolates at a 90% similarity level. As previously found with PS1, PS2 clustered environmental isolates VP1-VP4 together and VP6-VP9 together into two groups (RT 12 and 13 respectively) with VP10 in a distinct RT of its own. As with PS1, isolates Vib1-Vib3 and Vib5 (RT 17) grouped separately to Vib6 - Vib10 (RT 09) from the Southampton environmental isolates (Fig 8.12). Environmental isolates from Spain fell into four types (RT 04, 05, 07 & 08) and the remaining environmental isolates from Dorset, where clustered, fell into RAPD Types 01, 03 and 06 (Fig. 8.12).

8.3.5 Reproducibility of RAPD-PCR

A comparison of repeat RAPD-PCR with six isolates is shown in Figure 8.13. Most of the bands were present in repeat PCRs (A – F) with variability found in both the presence or absence of weak bands and the intensity of what appeared to be the same amplicon (Fig 8.13). For example, the 610 bp amplicon was present in all six repeats of type strain 1902 but intensities were variable (Fig. 8.13: Lanes A & B; isolate 1902). At least six faint bands were absent in Lane B (reflecting Fig. 8.4) in comparison with the remaining five tests where most of these bands could be identified (Fig. 8.13: isolate 1902). Similarly, repeat tests with isolate 2436, VP345, Vib2 and 1021/5 identified most of the prominent bands with the presence or absence of 2 or 3 very faint bands differentiating amongst the repeats (Fig. 8.13). A comparison of repeats with VP2 also demonstrated the degree of reproducibility of RAPD-PCR bands. Whereas Lane B (Fig. 8.13: VP2) showed the absence of minor bands, certain bands such as the 610 bp amplicon, in contrast, appeared brighter. Minor bands were ignored in BioNumerics cluster analyses.

8.3.6 Comparison of RAPD-PS1 and RAPD-PS2

RAPD-PS1 appeared more discriminating than RAPD-PS2. PS1 was able to differentiate two clinical isolates (219 and 224) that existed in the same group in PS2. Similarly, PS1
differentiated between three Spanish environmental isolates (1022/10, 1023/1 and 1023/3) and three UK environmental isolates (VP349, VP350 and VP361) that were grouped together in Pulse Types 03 and 06 with PS2. Additionally, PS2 was able to differentiate isolates that were grouped together in RAPD-PS1. For example, clinical isolate E154482 was differentiated from the other two clinical isolates in RT 21 of PS1. A comparison of the two typing methods where the values from the similarity matrices were plotted on x and y axes showed a congruence of 56.6% with Pearson’s correlation showing some concordance between the two primer pairs (Fig. 8.14).

8. 3. 7 Differentiation of clinical and environmental isolates

It was possible to differentiate clinical isolates from environmental isolates based on the RAPD patterns shown (Figs. 8.10 and 8.12). The two markers amongst clinical isolates, Band X and Band Y, account for 50% of the clinical isolates studied. Band X was approximately 910 bp in size and Band Y was approximately 605 bp length (Figs. 8.9, 8.10 and 8.11). Isolates were considered positive for bands X and Y if they appeared distinct and intense. However, with a large number of RAPD types, there was no single unique banding pattern or the presence or absence of a band that could clearly distinguish pathogenic *V. parahaemolyticus*. 
Figure 8.1: RAPD single primer (P2) screening of *V. parahaemolyticus* environmental isolates, VP350 and VPSCI. Lanes correspond to the following: 1 and 10; Control DNA (*E. coli* BL21 and C1a respectively). Lanes 2, 3, 4 and 5; VP350 (12 ng DNA) with 0, 10, 20 and 30 pmol primer respectively. Lanes 6, 7, 8 and 9; VPSCI (25 pmol primer) with 0, 15, 30, 45 ng of template respectively. Lanes M, 100 bp Ladders (Gibco). Arrows indicate non-specific PCR products typical of RAPD reactions.

Figure 8.2: RAPD double primer screening of *V. parahaemolyticus* environmental isolate, VP350. Primers P1 to P6 were used in pairs for RAPD PCR. Lanes M, 100 bp Ladders (Gibco). Lane C1 and C2 are no DNA template and no primer controls respectively. Primer PS1 and PS2 were used for further studies.
Figure 8.3: RAPD analysis of a panel strain of 16 representative clinical and environmental isolates of *V. parahaemolyticus* with primers P2 and P3 (PSI). Lanes 1 and 2: no template and no primer controls respectively. Lanes 3 to 20 correspond to the following isolates: 3: *E. coli* BL21 (control DNA); 4: *V. anguillarum* CM104; 5: VPSCI; 6: VPEC1; 7: VP939; 8: VP377; 9: VP350; 10: VP345; 11: VP7; 12: VP4; 13: VP3; 14: VP2; 15: VP1; 16: 5421; 17: 2436; 18: 6316; 19: 1021/5; 20: NCIMB type strain 1902. Lanes M1 and M2, 1Kb and 100 bp ladders respectively, with the sizes of selected markers indicated by the arrows.

Figure 8.5: RAPD banding patterns of UK and Spanish environmental isolates of *V. parahaemolyticus* with PSI. Lane 1: no template control. Lanes 2 to 20 correspond to the following isolates: 2: NCIMB reference strain 1902 (internal control); 3: 1021/5; 4: 1022/10; 5: 1023/1; 6: 1023/3; 7: 1024/5; 8: 1103/9; 9: VP378; 10: VP331; 11: VP345; 12: VP346; 13: VP349; 14: VP350; 15: VP361; 16: VP377; 17: *Proteus mirabilis*; 18: *Salmonella typhimurium*; 19: *E. coli* EC5; 20: *E. coli* C1a. Lanes M, 100 bp ladders with the sizes of selected markers indicated by the arrows.

Figure 8.6: RAPD banding patterns of Japanese clinical isolates and UK environmental isolates of *V. parahaemolyticus* with PSI and related species. Lane 1: no template control. Lanes 2 to 20 correspond to the following isolates: 2: NCIMB reference strain 1902 (internal control); 3: 2053; 4: 2436; 5: 5421; 6: 6134; 7: 6316; 8: *V. alginolyticus* 2341; 9: Vib3; 10: Vib5; 11: VP416; 12: VP491; 13: VP939; 14: VPEC1; 15: VPS1; 16: *V. anguillarum* CM104; 17: *Escherichia aerogenes* EA5; 18: *Klebsiella aerogenes* KA5; 19: *Pseudomonas aeruginosa* PA1; 20: *E. coli* BL21. Lanes M: 100bp ladders with the sizes of selected markers indicated by the arrows.
Figure 8.7: RAPD banding patterns of environmental isolates of *V. parahaemolyticus*, *V. cholerae* and related species with PSI. Lane 1: no template control. Lanes 2 to 20 correspond to the following isolates: 2: NCIMB reference strain 1902 (internal control); 3: 8042; 4: VC1; 5: VC2; 6: VC3; 7: VC4; 8: VC5; 9: *V. alginolyticus* VA430; 10: VP1; 11: VP2; 12: VP3; 13: VP4; 14: VP6; 15: VP7; 16: VP8; 17: VP9; 18: VP10; 19: NCTC reference strain VP10884; 20: *V. mimicus* 499. Lanes M: 100bp ladders with the sizes of selected markers indicated by the arrows.

Figure 8.8: RAPD banding patterns of environmental and clinical isolates of *V. parahaemolyticus* and related species with PSI. Lane 1: no template control. Lanes 2 to 20 correspond to the following isolates: 2: NCIMB reference strain 1902 (internal control); 3: *V. fluvialis* NCTC type strain 11327; 4: Vib1; 5: Vib2; 6: Vib3; 7: Vib4; 8: Vib5; 9: Vib6; 10: Vib7; 11: Vib8; 12: Vib10; 13: *V. vulnificus* KIDBVV; 14: *V. vulnificus* 11067; 15: *Listonella damselae* LD365; 16: E154482; 17: E155855; 18: E168143; 19: 2436; 20: 6134. Lanes M: 100bp ladders with the sizes of selected markers indicated by the arrows.
Figure 8.9: A unique RAPD marker (Band X) amongst clinical strains of *V. parahaemolyticus*. Clinical isolates correspond to the following lanes:- 1: NCIMB reference strain 1902; 2: 220; 3: 221; 4: 222; 5: 223; 6: 225; 7: 226; 8: 227; 9: E155855; 10: 2053. Amplicon also present in isolates 5421 and E168143 (Fig. 8.6: Lane 5 and Fig. 8.8: Lane 18 respectively). O and K serotypes are indicated in each lane. Lanes M: 100 bp ladders with the sizes of selected markers indicated by the arrows.
Figure 8.13: Reproducibility of RAPD fingerprinting profiles in six isolates of *V. parahaemolyticus*. Lanes A to F are comparisons of independent RAPD-PCR and gel runs from Figures 8.3 to 8.8 respectively. Reference arrow on the left shows the position of a 610 bp amplicon that appeared in isolates 1902, 2436, VP2, Vib2 and 1021/5 indicating relative migration of bands on each gel. Isolate VP345 is marked at a 500 bp amplicon. All band sizes are approximate.
Figure 8.14: Concordance of stratification by RAPD-PS1 and RAPD-PS2 for 56 isolates of *V. parahaemolyticus*. Each dot represents corresponding similarity values for two isolates obtained by both primer sets given on the x and y axes. The congruence between the two primer pairs as calculated with the Pearson coefficient was 56.5%. Data showed a linear regression fit of 68.8%. 

\[ r = 68.8\% \]
8.4 Discussion

This chapter examined the feasibility of the RAPD technique to differentiate clinical and environmental isolates of *V. parahaemolyticus*, as well as assess the reproducibility and reliability of the method used. Using pairs of RAPD primers produced a more complex banding pattern than single primer alone, as this increases the chances that two priming sites are recognised and are sufficiently close to result in a PCR amplification product (Welsh & McClelland, 1990). The method was easy to execute and the resulting large number of amplicons allowed intra-species differentiation in isolates that were previously grouped together using 16S-23S ITS and tDNA-ILPs spacer region analyses.

The method that was employed also allowed *Vibrio* species to be clearly differentiated from one another with the exception of one isolate, 6316, that could not be grouped with other members of *V. parahaemolyticus*. This isolate has repeatedly shown unique banding profiles in Chapters 5, 6 and 7 with a fingerprinting profile that is characteristic of *V. mimicus*. Further tests would be required to accurately speciate this isolate.

RAPD is a widely used method that is known to be sensitive in detecting small changes in the genome between isolates and is used to determine genetic relatedness both amongst species and sub-species. The use of a wide range of RAPD primers have been reported to type *V. parahaemolyticus*, but often typing experiments are compromised by the relatively small number of bands that are used to discriminate within a typing experiment. This was shown by Maluping and colleagues and further supported by this work (Section 8.3.1 and Fig. 8.1) (Maluping et al., 2005). This study concentrated on four primers P1, P2, P3 and P5 that are widely used in typing experiments, but used in pairs to develop and optimise a RAPD method that appeared reproducible and that could cluster distinct clinical and environmental groups of isolates.
8.4.1 Typing of *V. parahaemolyticus* based on PS1

Initial primer screening experiments suggested that primers P2 and P3 may produce banding patterns of suitable complexity. Up to nine common bands were amplified continually in most isolates of *V. parahaemolyticus*, and this allowed confirmation of relatedness. Several of these bands were also common in related *Vibrio* species suggesting that this RAPD method may 1) be a helpful tool to differentiate *V. parahaemolyticus* from other vibrios and 2) be able to differentiate between members of other species such as clinically significant and clonally related *V. cholerae*.

Of the 56 isolates of *V. parahaemolyticus* studied, PS1 differentiated the isolates into 30 RAPD Types (RTs) of which 19 were environmental only, eight were clinical only, a single RAPD Type consisting of both on an environmental and clinical isolate, and two RAPD types accounted for the two reference type strains. This data suggests that both clinical and environmental isolates were discriminated almost equally with PS1, and that RAPD can differentiate clinical isolates that have previously shown to be a more homogeneous group (Chapters 4, 6 and 7). The sensitivity of the RAPD technique has been widely documented (reviews by Atienzar & Jha, 2006; Yakubu et al., 1999). Clinical isolates of 219, 224, 2436 (as well as 6316) that fell into their own RAPD types and the clustering of a Kanagawa positive (haemolytic) environmental isolate with a Japanese clinical isolate is in agreement with PFGE findings (Chapter 5).

Whilst the RAPD method is sensitive, there are several criticisms of the technique. For example, whilst a complex banding profile with two primers was favoured in this study, it is likely this contributed to the presence of additional faint bands and possibly reducing the intensity of others (Fig 8.13). Weak bands have been shown to be irreproducible in most cases as identified in repeat tests. Whilst BioNumerics settings allowed the option of omitting very faint bands in cluster analysis it did not account for varying band intensities.
inherent in the RAPD method that were used in the curve calculations. This may provide at least one explanation why the same method with two different sets of random primers failed to give a high concordance (i.e. a congruence of greater than 70%) even though isolates in both dendrograms initially appeared grouped similar from visual inspection. A further explanation for discordance using the same method but a different set of primers could be attributed to the GC content of the primers. Whereas primers P2, P3 and P5 were all 60% GC, primer P1 had a 70% GC ratio (Table 8.1). Invariably, such differences in % GC are likely to result in considerable changes to primer and template DNA interactions. A complex gel banding pattern also meant that agarose gels required adequate running times to obtain good resolution. In practice, consistency was difficult to achieve between different gel runs even with the use of a tracking dye and thus further reliance on BioNumerics normalisation procedures. The use of additional markers within the gel would therefore facilitate more accurate normalisation of bands and to adjust for possible gel smiling.

8.4.2 Typing of *V. parahaemolyticus* based on PS2

It was necessary to validate groupings and clusters determined with PS1 and therefore the second primer set PS2 utilised Primers 1 and 5. Although a smaller number of bands were produced, a fair degree of concordance was shown between the two typing experiments. Isolates could be characterised within a cluster, forming smaller groups or identified as its own type. For example, the lower number of bands produced in PS2 meant that the ability to discriminate was less than that of PS1. However, although PS1 discriminated 10 isolates that were grouped in PS2, PS2 also discriminated three isolates that were grouped in PS1; therefore, the results of the two sets of RAPD primers were complementary rather than contradictory. Chapter 10 addresses the evaluation of RAPD typing with other molecular typing systems employed.
8.4.3 A marker for pandemic serovars?

One of the most interesting findings of typing *V. parahaemolyticus* with two primers was the identification of a band in 10 of the clinical isolates (Fig. 8.9) that was present using both primer sets, PS1 and PS2. These distinct bands were found in isolates of four serovars (O1:KUT, O3:K6, O3:KUT and O5:K68) from three different geographical regions, but appeared faint in many of the environmental isolates. PFGE grouped them all under one cluster at 76% similarity that consisted of Pulse Types 18, 19, 20 and 21 (Fig 5.11) but did not manifest as a singularly identifiable band. RAPD dendrogram analysis was also able to differentiate these isolates into RAPD types 10, 21 and 24 with PS1, and RAPD types 15, 22 and 23 with PS2. In order to confirm the identity of these bands, and whether they are truly different to those from environmental isolates, it would be necessary for further repeats, as band intensities mentioned earlier, are variable in the RAPD method. Furthermore, more reliable band sizing techniques, such as polyacrylamide gel electrophoresis, would be necessary for more accurate analysis. This should be followed by cloning and sequencing of bands to investigate the 910 bp and 610 bp RAPD markers identified in clinical isolates. It has also been proposed that the arbitrary bands produced in RAPD do not reflect real and relevant population diversity and as such it would be difficult to monitor the stability of the typing system during the time frame of the investigations (Burr et al, 1998).

8.4.4 Comparison of single and two-primer RAPD methods

RAPD typically employs the use of short 10-mer primers. Several workers have used the 10-mer primers P1, P2 and P4 singly to demonstrate clonal relationships in O3:K6 isolates of *V. parahaemolyticus* (Matsumoto *et al.*, 2000; Okuda *et al.*, 1997a; 1997b). Of all these primers used, P1 showed the most bands and the presence or absence of one or two bands was used as criteria to differentiate the old and new O3:K6 isolates; those that appeared before and after 1996 respectively – the latter referring to the more virulent O3:K6 types.
Additionally, the use of Primer 2 showed even fewer total bands and, again, the presence or absence of one or two bands differentiated the two groups in a sample of eight ‘old’ O3:K6 and nine ‘new’ O3:K6 isolates (Matsumoto et al., 2000). Thus the use of a two-primer method described here that generated up to 20 bands may be better able to differentiate clonally related isolates. It is unfortunate that the study did not contain a significant number of comparative old and new O3:K6 types to test this claim.

PCR product size with single primer P1 has been shown to range in size from approximately 0.6 - 6.5 kbp (Okuda et al., 1997b) whereas this work has demonstrated that most of the PCR products could be resolved within 0.1 and 2.0 kbp. This is to be expected as the distance between primed sites is likely to be shorter when two different arbitrary primers are available than a single primer alone (Welsh & McClelland 1991b). Shorter amplicons may also amplify more efficiently because of the shorter extension times required and the increased likelihood that DNA polymerase will complete strand synthesis (Hopkins & Hilton, 2001). Therefore the use of two primers would further increase efficiency to subtype V. parahaemolyticus, by minimising times needed for agarose gel electrophoresis. The use of a third primer might be advantageous, but in one reaction this has been shown to be inappropriate for typing purposes as PCR product sizes were significantly smaller than one- or two-primer PCR reactions and required the use of polyacrylamide gels to resolve smaller fragments (Hopkins & Hilton, 2001). This may not be practical for routine typing purposes to process a large number of samples. Additionally, the presence of too many bands in a typing system increases the complexity of the visual interpretation leading to reliance on more sophisticated band analysis software.
8.4.5 Data analyses

As with previous chapters, the quantitative assessment of fingerprinting profiles was necessary throughout the analysis of RAPD fingerprints. One of two methods usually employed are either a densitometric curve based approach (as used in this chapter and chapters 6 and 7) or a band based approach as used in Chapter 5. Whereas the binary nature of distance matrices in the band based approach scores bands as either present or absent, a densitometric curve analysis method takes into account the intensities of different bands produced by RAPD. Despite, variation in band intensities shown in the present study, this method has been shown to be more reliable in producing RAPD clustering than the band based Dice method (Duarte et al 2005). Additionally, the availability of fingerprint analysis software facilitated the process of determination of groupings. Nonetheless, much caution is still needed in the interpretation of RAPD data. For example, the co-migration of bands may be a result of amplicons of the same size, yet generated from different regions of the genome. If the presence of these bands is used to infer similarity between isolates, then clearly two identical RAPD patterns may not necessarily imply they are the same strain. Therefore, when analysing large amounts of data that are used to construct a dendrogram that infers phylogenetic relationships, careful interpretation of banding patterns is required. Thus, the use of a second primer set PS2 in this chapter was necessary to confirm groupings obtained, and the larger number of amplicons in a more complex banding pattern may serve to reduce errors from co-migration of bands.

8.4.6 General considerations of a RAPD typing system

The main advantages of the RAPD method are its relative simplicity to set up and use. However, several problems may potentially hamper its use and these have been the subject of criticism of the technique. As mentioned, these include the lack of reproducibility between laboratories due to sensitivity to changes in reagents, protocols and machines, the non-universal use of primers and RAPD markers as well as the aforementioned method of
data analysis used (Chang et al., 2007). However, when the same method was used with different thermocyclers, comparable results for *V. parahaemolyticus* have been achieved (Matsumoto et al., 2000). For example, RAPD profiles generated with a “Hybaid HB-TRIL” and “Zymoreactor II – AB-1820” thermocyclers were used to reliably compare strains from Bangladesh with those isolates from many other countries to study the spread of pandemic O3:K6 clones (Matsumoto et al., 2000). In this chapter, internal controls using the DNA of several isolates as well as NCIMB reference strain 1902 were used. These results have shown some reproducibility and consistency in banding profiles in independent PCR reactions (Figs. 8.4 to 8.8), however more thorough inter-laboratory comparisons are required to assess the reproducibility of this two-primer method.

Despite the criticisms, the initial concerns about the reproducibility of RAPD subtyping have been partially addressed by the development of more robust RAPD protocols, and in particular, standardised RAPD reagents and ‘analysis beads’. Therefore, a comparison of RAPD with and without the use of analysis beads would be interesting. The RAPD method developed in this chapter allowed discrimination equal to that of PFGE, yet requiring a fraction of the time to execute. It is estimated that a total of 5 hours of DNA/PCR preparation, thermocycling and gel running times gave data comparable to the sensitivity of PFGE with no concerns of untypeability. This would be more cost and time efficient than the recently described PulseNet USA standardised pulsed-field gel electrophoresis protocol for subtyping of *V. parahaemolyticus* (Parsons et al., 2007) Analysis on a larger number of samples using primers P2 and P3 would confirm the general usefulness of this method and, in particular, to determine if the markers can be used to distinguish clinical isolates.
Chapter 9

VARIATION IN THE TYPE III SECRETION SYSTEM AMONGST CLINICAL AND ENVIRONMENTAL ISOLATES OF VIBRIO PARAHAELOMYCITUS

9.1 Introduction

The type III secretion system (TTSS) is an apparatus used by several Gram-negative bacteria to secrete and translocate virulence effectors across the bacterial inner and outer membranes directly into the cytosol of host eukaryotic cells (Cornelis & Van Gijseghem, 2000; Hueck, 1998; Ghosh, 2004). The TTSS is found in both animal and plant pathogens and is composed of more than 20 proteins that form a macromolecular assembly (TTSS1, Fig. 9.1). The proteins of the TTSS apparatus are highly conserved among bacteria that possess the system (Tampakaki, et al., 2004). In contrast, the effector proteins secreted via the TTSS are not conserved among those bacteria, and hence, the biological effects vary widely in target host cells (Hueck, 1998; He et al., 2004). Genes encoding the TTSS apparatus are generally found on pathogenicity islands (PAIs) or virulence plasmids (Hueck, 1998; Winstanley & Hart, 2001). The TTSS has been shown to be especially important in the pathogenicity of Yersinia spp., Burkholderia spp., Salmonella spp., Shigella flexneri, and enteropathogenic Escherichia coli (Hueck, 1998; Winstanley & Hart, 2001).

Genome sequencing of the clinical V. parahaemolyticus isolate RIMD2210633 revealed that the bacterium possesses two sets of genes for a TTSS, termed TTSS1 and TTSS2, located on chromosomes 1 and 2 respectively (section 2.1.2) (Makino et al., 2003). The gene organization of TTSS1 was found to be very similar to that of the TTSSs reported for Yersinia species. In contrast, TTSS2 is more similar to that found in non-O1 and non-O139
*V. cholerae* pathogenic strains and seems to contain at least essential genes for protein secretion (Dziejman *et al.*, 2005). The distribution of those genes among various *V. parahaemolyticus* strains and the GC content of the DNA regions containing TTSS1 or TTSS2 suggested that TTSS1 is intrinsic in the species while TTSS2 has a feature of laterally transferred DNA (Makino *et al.*, 2003). Other bacterial species also possess two or more TTSSs. For example, multiple TTSSs can be found in *S. typhimurium* and *Burkholderia* spp. (Hueck, 1998; Winstanley & Hart, 2001). Enterohemorrhagic *E. coli* O157:H7 has been known to possess two TTSSs termed LEE and ETT2 (Perna *et al.*, 2001). LEE is involved in the formation of attaching and effacing lesions in epithelial cells whereas the function of ETT2 remains unknown (Ideses *et al.*, 2005). Except for the case of the TTSSs in *Salmonella* spp., the roles of multiple TTSSs in a single bacterium have not yet been elucidated. In *V. parahaemolyticus*, TTSS1 was found in all of the strains tested while TTSS2 was present only in the KP-positive strains (Makino *et al.*, 2003).

The present study was conducted to:

1. develop multiplex PCRs to assess the distribution of both TTSS systems in clinical and environmental isolates that possess the gene *tdh* and shown to be K+ or K- (Chapters 3 and 4)
2. examine variation in both TTSS1 and TTSS2 in *V. parahaemolyticus* isolates by the development of probes specific to genes of TTSS1 and TTSS2 and DNA hybridisation assays
3. assess the relationships between clinical and environmental isolates possessing TTSS1 and TTSS2 systems with phylogenetic clusters obtained from typing experiments with a view to discrimination of potentially pathogenic isolates.
9.2 Materials and Methods

9.2.1 PCR detection of TTSS1 and TTSS2 genes

Using sequence information from isolate RIMD2210633 (Makino et al., 2003), three pairs of PCR primers spanning each of the TTSSs were designed with the aid of DNA-sis (Hitachi Software) and Amplify software (Engels, 1993). The primers were targeted to three genes on TTSS1 and two genes on TTSS2 as well as a region flanking the TTSS2 genes representing the \textit{V. parahaemolyticus} pathogenicity island (Vp-PAI) (Izutsu et al., 2008) (Table 9.1, Fig. 9.1). Primers consisting of 24 bases were designed on the basis of a 50% GC ratio, the presence of 2-3 guanine or cytosine bases at the 3' end of the primer where possible, a lack of secondary structure and primer dimer formation, and their proximity in relation to the desired target sequence (Table 9.2).

\textbf{Table 9.1: TTSS gene targets in \textit{V. parahaemolyticus} used in this study.} Genes \textit{vcrD1} & \textit{vscC1} are components of the TTSS1. Gene \textit{vcrD1} has 74\% identity to \textit{Yersina} sp. \textit{yscD} (encoding the largest protein). Gene \textit{vscC1} has 48\% identity to \textit{Yersinia} sp. \textit{yscC}. An absence of these genes is likely to result in defective protein secretion via the TTSS1.

<table>
<thead>
<tr>
<th>Region</th>
<th>Gene number</th>
<th>Designation of probe</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTSS1</td>
<td>VP1662 (vcrD1)</td>
<td>Probe A</td>
<td>Low calcium response protein. TTSS structural protein</td>
<td>Park \textit{et al.}, 2004b</td>
</tr>
<tr>
<td></td>
<td>VP1680 (vp1680)</td>
<td>Probe B</td>
<td>'Cytotoxic effector' protein</td>
<td>Ono \textit{et al.}, 2006</td>
</tr>
<tr>
<td></td>
<td>VP1696 (vscC1)</td>
<td>Probe C</td>
<td>TTSS structural protein</td>
<td>Park \textit{et al.}, 2004b</td>
</tr>
<tr>
<td>TTSS2</td>
<td>VPA1321 (vpa1321)</td>
<td>Probe D</td>
<td>Cytotoxic necrotizing factor \textit{V. parahaemolyticus} PA1</td>
<td>Makino \textit{et al.}, 2003</td>
</tr>
<tr>
<td></td>
<td>VPA1339 (vscC2)</td>
<td>Probe E</td>
<td>Putative TTSS structural protein</td>
<td>Dziejman \textit{et al.}, 2005</td>
</tr>
<tr>
<td></td>
<td>VPA1355 (vcrD2)</td>
<td>Probe F</td>
<td>Putative TTSS structural protein</td>
<td>Dziejman \textit{et al.}, 2005</td>
</tr>
</tbody>
</table>

The reaction conditions for PCR amplification of \textit{V. parahaemolyticus} TTSS loci in separate reactions are described in section 3.6 except that 25 \textmu l rather than 50 \textmu l reaction volumes were used and consequently half the stated volume of each reagent was added. Details of the primers used are provided in Table 9.2. Optimised PCR cycling conditions were 2 min at 94\degree C followed by 30 cycles of 1 min at 94\degree C, 45 sec at 65 \degree C and 90 sec at
72°C followed by a final extension time of 5 min at 72°C. PCR products were visualised by gel electrophoresis of 6 - 12 μl sample volumes on 1.5% agarose gels, as described in section 3.8.

9.2.2 Development of Multiplex PCRs

Two multiplex PCRs (M-PCR) were developed using the 6 primer pairs described in Table 9.2. A maximum of three loci per reaction were amplified. The PCR reactions were performed in a 50 μl volume as described in section 3.6; primers for the TTS1 regions used an equimolar 20 pmol concentration with the same PCR cycling conditions as in section 9.2.1. Amplification of TTSS2 regions used an equimolar 30 pmol concentration of each of the primers. The M-PCR products were visualised by electrophoresis of 10 μl volumes of the reactions in a 1.5 % agarose gel as described in section 3.8.
Table 9.2: PCR primer sequences used to detect TTSS1 and TTSS2 regions

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<thead>
<tr>
<th>Region</th>
<th>Gene number</th>
<th>%GC</th>
<th>Amplicon size (bp)</th>
<th>Primer designation &amp; sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTSS1</td>
<td>VP1662</td>
<td>53.9</td>
<td>458</td>
<td>VP1662+764: TCACCGCTGGTATTATCGTTACGC</td>
</tr>
<tr>
<td></td>
<td>(2127bp)</td>
<td></td>
<td></td>
<td>VP1662-1221: GAGATACAAACGCACAGTTACTCT</td>
</tr>
<tr>
<td></td>
<td>VP1680</td>
<td>53.3</td>
<td>183</td>
<td>VP1680+613: GCCGAAGCGTATCATCATCAACTC</td>
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<tr>
<td></td>
<td>(1479bp)</td>
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<td></td>
<td>VP1680-795: CACAGAGCTTACACAAACGTACC</td>
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<tr>
<td></td>
<td>VP1696</td>
<td>52.0</td>
<td>415</td>
<td>VP1696+655: ATTGCTTCGGTTATGAGCCGTTG</td>
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<tr>
<td></td>
<td>(1899bp)</td>
<td></td>
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<td>VP1696-1069: AACTCTGACCACCTGCCCAGTGA</td>
</tr>
<tr>
<td>TTSS2</td>
<td>VPA1321</td>
<td>36.9</td>
<td>485</td>
<td>VPA1321+195: GGTTAGTGAATCCAAACCCGAC</td>
</tr>
<tr>
<td></td>
<td>(1107bp)</td>
<td></td>
<td></td>
<td>VPA1321-679: TGTCGCTGCATGTCAACCAACAG</td>
</tr>
<tr>
<td></td>
<td>VPA1339</td>
<td>41.6</td>
<td>272</td>
<td>VPA1339+398: GACACTCGCTGTGGTTCTCAGGTA</td>
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<tr>
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<td>(1464bp)</td>
<td></td>
<td></td>
<td>VPA1339-669: GAAGCGCTGATGTAGCTCTTC</td>
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<tr>
<td></td>
<td>VPA1355</td>
<td>40.6</td>
<td>388</td>
<td>VPA1355+747: GCATGTGGGTCTATTTGACACG</td>
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<tr>
<td></td>
<td>(1884bp)</td>
<td></td>
<td></td>
<td>VPA1355-1134: TACGACACTGCGAGTGTCAAG</td>
</tr>
</tbody>
</table>

1Primers based on published sequence of clinical strain RIMD2210633 (Makino et al., 2003)
Figure 9.1: Genetic organisation of TTSSs from *V. parahaemolyticus* RIMD2210633 showing targets for PCR and DIG labelled probes. Probes A to Probe F targeted to approximate regions of the TTSS regions. Probe D lies outside of the TTSS2 regions identified as a *V. parahaemolyticus* pathogenicity island (VpPA1), indicated by flanking black/orange lines (Izutsu *et al.*, 2008). Grey arrows show annotated TTSS genes where known. Unshaded arrows indicate hypothetical genes (adapted from Ono *et al.*, 2006 & Dziejman *et al.*, 2005).
9.2.3 Production of TTSS1 and TTSS2 specific DIG labelled probes

PCR products corresponding to six of the TTSS regions from the genome of NCIMB UK type strain 1902 was used to develop digoxigenin (DIG) labelled probes and were designated Probes A to Probes F, according to PCR amplification regions (Fig 9.1). Details of probes labelled are shown in Tables 9.1 and 9.2. Additionally, three PCR products corresponding to the TTSS2 regions of an O3:K6 clinical isolate (220) that gave a strong PCR signal was also used to develop probes.

The random primed method of labelling of DNA probes was performed according to the protocol provided in the Roche instruction manual ‘DIG DNA Labeling and Detection Kit’. Details of all reagents referred to in this section can be found in Appendix I. PCR products were purified using the ‘High Pure PCR Product Purification Kit’ (Roche) and DNA quantified using the methods described in section 3.4. The reactions were performed in 0.5 ml Eppendorf tubes, each containing 0.4 - 1 μg of purified PCR product, 2 μl of hexanucleotide mixture (stock concentration 1.56 mg/ml), 2 μl dNTP labelling mixture; the mixture was made up to 19 μl with the addition of molecular grade water and then 1 μl of Klenow enzyme (2 U/μl), was added. The tubes were vortexed, centrifuged briefly at room temperature (13000-16000 x g, 5 s) and incubated at 37°C for at least 1 h, after which 2 μl of 0.2M EDTA solution was added to each tube to stop the reactions.

9.2.4 Preparation of dot-blots

Genomic DNA was transferred onto positively charged nylon membrane by using a Minifold I 96-well dot-blot system (Schleicher & Schuell, Germany) using the manufacturer’s instructions. A volume corresponding to 100 ng DNA was diluted to 100 μl with molecular grade water, boiled for 10 min and kept on ice. An equal volume of freshly prepared NaOH (1M) was added to the samples and incubated at room temperature
for 20 min before transferring onto the nylon membrane. After drying for 10 min, the membrane was removed from the apparatus and DNA UV cross-linked for a further 5 min (Chromato-Vue cabinet, Module: CC-60 UVP Inc), followed by incubation in neutralising solution for 30 min. Membrane was rinsed twice in 2 x SSC and air dried and hybridised.

9.2.5 DNA-DNA Hybridisation

DIG labelled probes were hybridised in DIG Easy Hyb solution (Roche) according to manufacturer’s instructions at temperatures of 65 - 68°C. Briefly, 20 ml of DIG Easy Hyb solution was preheated to hybridisation temperature in a roller bottle and the blot was added and left for 20 min for a prehybridisation wash. DIG labelled DNA probe (prepared as in Section 9.2.3) was denatured and added to a preheated 5 ml of Easy Hyb solution at a final concentration of 10 ng/ml. Prehybridisation solution was discarded and replaced with the probe/DIG Easy Hyb mixture. Probe DNA was hybridised overnight in the roller bottles at the appropriate hybridisation temperature. Post hybridisation stringency washes were performed twice in 20 ml of 2x SSC containing 0.1% SDS for 5 min at 20°C, with two further washes in 10 ml of 0.5x SSC containing 0.1% SDS at 68°C for 15 min in roller bottles.

9.2.6 Immunological detection of DNA-DNA hybrids

Details of all reagents and buffers referred to in this section can be found in Appendix 1. Immunological detection of DNA hybrids was performed according to the protocol provided in the Roche instruction manual ‘DIG DNA Labelling and Detection Kit’. Blots prepared as previously described were submerged for 1 min in 200 ml of buffer number 1 with constant gentle agitation, and then placed in 200 ml buffer number 2 for 30 min. The membranes were rinsed with buffer 1 then placed in a solution containing anti-digoxigenin antibody conjugated to alkaline phosphatase diluted 1:10,000 in buffer 1 for 2 h at room temperature. Any unbound antibody was subsequently removed by washing the
membranes twice in buffer 1 for 15 min. The membranes were equilibrated for 2 min in 100 ml buffer 3 and then immersed in 70 ml of developing solution. The development reaction was allowed to proceed in the dark until a colour precipitate formed. The reaction was terminated by washing the membrane several times with TE buffer. Images of the developed membranes were captured onto a digital camera (Sony Cybershot DSC-10) with the macro settings enabled and the intensities of hybridisation signals were scored 0 to 3.

9.2.7 Sequencing and analysis of TTSS genes

Both strands of PCR products corresponding to all six TTSS loci derived from NCIMB UK type strain 1902 were sequenced by MWG Biotech AG. For comparative purposes, amplicons derived from two additional isolates were also sequenced (O3:K6 clinical isolate 220 and environmental isolate VP416) These sequences, together with the published genome sequence of *V. parahaemolyticus* strain RIMD2210633, were aligned using the online programs MultAlin, Artemis, ACT and Blastn 2. (Carver et al., 2005; Corpet, 1988; Makino et al., 2003; Tatiana et al., 1999). Sequences were also compared with those from other organisms on the GenBank database using the gapped BLAST program at http://blast.ncbi.nlm.nih.gov (Altschul et al., 1990).

9.3 Results

9.3.1 PCR primers targeted to the TTSS loci

PCR primers based on the published genome sequence of *V. parahaemolyticus* RIMD2210633 successfully amplified regions of both sets of TTSS genes in most of the 55 isolates of *V. parahaemolyticus* tested, as well as in some isolates of *V. cholerae*, *V. vulnificus* and *V. alginolyticus* (Figs. 9.2, 9.3 & 9.4). Most of the TTSS amplicons from *V. parahaemolyticus* appeared consistent in size, with various degrees of intensity. Sequencing of the PCR products of three isolates (1902, 220 and VP416) followed by
BLAST searches confirmed their identity as TTSS regions of the \textit{V. parahaemolyticus} genome (section 9.3.7).

An annealing temperature of 64°C proved to be the most reliable in the amplification of both TTSS1 and TTSS2 genes using the same cycling conditions (Fig 9.2). Two multiplex PCRs targeting the TTSS1 and TTSS2 regions respectively both amplified three loci of correct sizes (Table 9.2) in most cases. The bands of the TTSS2 regions appeared weaker in the multiplex PCR reactions and an increase of primer concentration by 5 pmol provided more consistent results (Fig 9.2).

**9.3.2 PCR amplification of genes from the TTSS1 region**

Of the 55 isolates of \textit{V. parahaemolyticus} tested, PCR was positive for all three loci of TTSS1 (representing the entire TTSS1 system) in 48 (87%) of the isolates. Of the remaining seven isolates, three clinical isolates (2436, VP10884 and 219) and two environmental isolates (VP331 and Vib1) PCR only amplified two out of the three genes (Figs. 9.3 & 9.4). Of the five isolates, only the amplicon corresponding to \textit{vpl680} showed a similar sized PCR product to the other 48 isolates (Figs. 9.3 & 9.4). In isolates 2436, VP331 and VP10884 PCR did not amplify \textit{vcrDJ} whereas in isolates 219 and Vib1 \textit{vscCJ} did not amplify. Repeated PCR on these isolates provided the same results. A further two environmental isolates VP345 and VP361 did not amplify any TTSS1 loci. The absence of the TTSS1 region was confirmed by DNA hybridisation (Table 9.4, section 9.3.5)

**9.3.3 PCR amplification of genes from the TTSS2 region**

The TTSS2 regions in both clinical and environmental isolates of \textit{V. parahaemolyticus} showed much more variability in intensity than the TTSS1 amplicons (Figs. 9.3 & 9.4). Genes near the TTSS2 region corresponding to \textit{vpa1321} were not amplified in two clinical isolates (2436 and 228) and 20 environmental isolates, accounting for 11% and 59% of the
two groups respectively. Clinical isolates that showed weak TTSS2 signals included 219, 221 and 224. Of the 15 environmental isolates that showed positive PCR for the TTSS2 region only two isolates (VP10 and Vib3) did not give an amplicon corresponding to vscC2 (Fig 9.4). In most cases apart from isolate VP416, PCR signals for the amplification of TTSS2 regions in environmental strains were weak (Fig 9.4). Again, the presence of the TTSS2 region was confirmed by DNA hybridisation (Table 9.4, section 9.3.5).

9.3.4 TTSS probes specificity in dot blots
DIG labelled probes A to E, developed from the specific PCR products of UK type strain 1902, hybridised successfully to most of the DNA samples from both clinical and environmental isolates (Figs 9.8 and 9.9). The hybridisation data compared well with PCR data and in most cases confirmed the presence of TTSS1 or TTSS2 regions in both clinical and environmental isolates of V. parahaemolyticus (Table 9.4). However, a weak hybridisation signal was obtained with some isolates that did not show the presence of TTSS regions by PCR. Additionally, a positive ‘halo effect’ in some of the samples was observed (Figs. 9.8 & 9.9). In such instances, the hybridisation signals were scored by the relative intensity of the halo as compared to the positive control. Only scores of 2 or 3, for the relative intensity of hybridisation signal, together with the presence of PCR signals were counted as positive for the presence of TTSS regions. There was no evidence to suggest that TTSS regions existed in multiple copies in any of the 56 isolates tested. Results are summarised in Table 9.4.

9.3.5 Dot blot analysis of TTSS1 genes vcrD1, vp1680 and vscC1
In support of the PCR results, dot blot hybridisation confirmed the presence or absence of TTSS1 regions in most of the clinical and environmental isolates of V. parahaemolyticus. Hybridisation signals with probe A (vcrD1) were present in all clinical isolates including isolate 2436 that was negative by PCR. However, five environmental isolates (VP6, VP7,
Vib10, 1024/5 and 1023/3) that had shown to be positive for the vcrD1 locus by PCR appeared negative or showed very weak signals in dot blot analysis. Furthermore, dot blot reliably confirmed the absence of vcrD1 in isolates VP331, VP345, VP361, VPEC1 and VPSC1 (Fig 9.6, Rows C & D).

Signals with probe B, representing the presence of the vp1680 locus, were present in all of the clinical isolates although signals were distinctly weaker in nine out of the 17 isolates (Fig 9.7, Rows A and B). However, three environmental isolates, (1022/10, 1024/5 and VP7) that were shown to be positive for the vp1680 locus by PCR appeared negative in dot blot analysis. Additionally, isolate VP345 that had shown to be PCR negative for vp1680 also showed a weak hybridisation signal.

Hybridisation signals with probe C, representing the presence of the vscC1 locus were present in all the clinical isolates and hybridisation signals were stronger than with Probe B (Fig 9.8, Probe C, Rows A and B). However, five environmental isolates (VP7, VP346, VP419, 1023/3 and 1024/5) that had shown to be positive for the vscC1 locus by PCR appeared negative in dot blot analysis. Furthermore, dot blot reliably confirmed the absence of vscC1 in isolates, VP345, VP361, VPEC1 and VPSC1 (Fig 9.8, Probe C, Rows C & D).

9.3.6 Dot blot analysis of TTSS2 genes vscC2, vcrD2 and vpa1321
Analysis of the hybridisation of probes specific to vscC2, vcrD2 and vpa1321 regions all gave similar results and in most cases confirmed PCR findings (Figs. 9.8 & 9.9). Clinical isolates that gave weak (219, 221 and 224) or no (2436 and 228) PCR signals for all three loci also gave weak dot blot hybridisation signals, confirming the presence of TTSS2 genes in all clinical isolates of V. parahaemolyticus examined. However, 11 environmental isolates that were negative by PCR for TTSS2 regions demonstrated weak to moderate

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hybridisation signals in comparison to the negative control isolates. Additionally, three environmental isolates (VP331, VP346 and Vib10) that appeared positive in PCR did not hybridise to probes D, E or F. All results are summarised in Table 9.4.

9.3.7 Conserved sequence identities of \textit{V. parahaemolyticus} TTSS genes

Multiple sequence alignments from three isolates together with RMID at all six loci used in this investigation revealed 100% congruence in nucleotide sequence in all three loci of the TTSS2 regions (Fig. 9.12). However, the sequences of the three TTSS1 regions showed some variation amongst the three isolates studied. Sequencing of PCR amplicons showed up to nine single nucleotide polymorphisms (SNPs) present within a fragment size of 458 bp for \textit{vcrD}1 (Fig 9.11). Additionally, \textit{vpl680} and \textit{vscC}1 showed 11 and 7 nucleotide substitutions respectively. These together with the TTSS2 sequence homologies account for between 97 and 100% similarity between all three isolates in the TTSS1 and TTSS2 regions. The SNPs do not translate into a different amino acid in \textit{vcrD}1, except for one codon where isoleucine is encoded in type strain 1902 instead of valine in isolates RIMD, 220 and VP416 (position 119; Fig. 9.11). In addition to the SNPs, \textit{vpl680} also exhibited nucleotide deletions at position 30 (in isolates 220, 1902 and VP416) and further deletions at positions 40, 50 and 147 in isolate VP416 with a resultant frameshift mutation in \textit{vpl680} (Appendix III; Fig. III.7).

9.3.8 Correlation of phylogenetic clustering of isolates with the presence of TTSS1 and TTSS2 genes

Four environmental isolates of \textit{V. parahaemolyticus} that were negative for TTSS1 region (VP345, VP361, VPEC1 and VPSC1) were clustered together and can be found in RAPD types 28, 29 and 30 (Chapter 8 & Fig. 9.10) with an additional isolate, VP331. Environmental isolates positive for the TTSS2 region generally fell into one group and two
sub clusters at a 76% similarity value (Fig 9.1). Of these clusters (indicated by red and blue in Fig 9.10), one group of 8 consisted entirely of environmental isolates and the other group consisted of a mixture of three environmental and four clinical isolates. A further three environmental isolates (VP2, Vib3 and Vib7) positive for the TTSS2 region were found scattered in various clusters (Table 9.4).

9.3.9 V. parahaemolyticus TTSS regions in other species

The PCR primers and probes developed from template of V. parahaemolyticus type strain 1902 were found to amplify PCR products in five other species tested including Vibrio mimicus (VM499), V. vulnificus (VV11067 and KIDBVV), V. cholerae (type strain 8042, VC3, VC4 and VC5), V. alginolyticus (2341) and V. anguillarum (Figs. 8.3, 8.4, 8.5, Table 8.3) Probes gave no hybridisation signals with Proteus mirabilis (PM5), Salmonella typhimurium (ST5), Escherichia aerogenes (EA5), Klebsiella aerogenes (KA5) and Pseudomonas aeruginosa (Fig 8.5)
Figure 9.2: PCR amplification at annealing temperatures of (A) 63°C, (B) 64°C and (C) 65°C for TTSS1 and TTSS2 genes in NCIMB type strain 1902 in single and multiplex PCR reactions. Lanes correspond to amplicons from the following, with approximate sizes: 1: M-PCR for all three TTSS1 products; 2: VPI662 (458 bp); 3: VPI680 (183 bp); 4: VPI696 (415 bp); 5: M-PCR for all three TTSS2 products; 6: VPA1321 (485 bp); 7: VPA1339 (272 bp); 8: VPA1355 (388 bp); 9: no template control; M: 100 bp markers with selected sizes indicated by arrow.
Figure 9.3: PCR of TTSS loci in clinical isolates of *V. parahaemolyticus* from Japan, UK and Norway. (A): amplicons of isolate 5421 derived from primers based on sequences of strain RIMD2210633. (B) and (C): For each isolate (as indicated above the lanes) a pair of lanes corresponds to TTSS1 and TTSS2 regions respectively. Lanes 1 - 10 Japan isolates with amplicons representative of all six loci; lanes 11 - 16 UK PHLS isolates; lanes 17 - 18 NCIMB reference strain VP10884; lanes 19 - 20 LD365 (*L. damselfa*); lanes 21 - 40 Norway isolates. Lane M corresponds to 100 bp ladders with selected markers indicated on the right.
Figure 9.4: PCR of TTSS loci in 34 environmental isolates of *V. parahaemolyticus* from UK and Spain. For each isolate (as indicated above the lanes) a pair of lanes corresponds to TTSS1 and TTSS2 regions respectively. Lanes 1 - 18 Southampton isolates; lanes 19 - 40 isolates from Dorset coast; lanes 31 - 56 Southampton isolates; lanes 57 - 58 unspeciated *Vibrio* sp. Vib 4; lanes 59 - 60 *V. mimicus*; lanes 61 - 62 NCIMB reference (clinical) strain; lanes 63 - 74 Spanish isolates; lanes 75 - 76 *V. cholerae*; lanes 77-78 *V. alginolyticus*; lanes 79-80 *V. fluvialis*. Lane M corresponds to 100 bp ladders.
Figure 9.5: PCR and hybridisation signals in *Vibrio* spp.
(A) Single primer and multiplex PCR signals of TTSS loci in *Vibrio* species with primers based on *V. parahaemolyticus* RMD2210633. Lanes 1-2: *V. cholerae* type strain 8042; lanes 3-6: *V. cholerae* environmental isolates; lanes 7-8: *V. vulnificus*; lanes 9-14: *V. vulnificus* VV11067 single primer PCR with all six primer sets; lanes 15-20: *V. vulnificus* environmental isolate KIDBVV single primer PCR with all six primer sets respectively. Lane M corresponds to 100 bp markers.
(B) Dot blot hybridisation signals (probe E) with isolates labelled above the dots. Isolates EC5, PM5, ST5, EA5, KA5 and PA1 are non vibrios.

Table 9.3: *Vibrio* species tested for TTSS genes of *V. parahaemolyticus*.

<table>
<thead>
<tr>
<th>Isolate Designation</th>
<th>Species</th>
<th>Location</th>
<th>Source</th>
<th>TTSS1</th>
<th>TTSS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1902</td>
<td><em>V. parahaemolyticus</em></td>
<td>PHL, UK</td>
<td>NCIMB Reference Strain</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8042</td>
<td><em>V. cholerae</em></td>
<td>PHL, UK</td>
<td>NCTC Reference Strain</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11327</td>
<td><em>V. fluvialis</em></td>
<td>Oslo, Norway</td>
<td>NCTC Reference strain</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2341</td>
<td><em>V. alginolyticus</em></td>
<td>Kyoto, Japan</td>
<td>Clinical</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VC1</td>
<td><em>V. cholerae</em></td>
<td>Southampton</td>
<td>Environmental</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VC2</td>
<td><em>V. cholerae</em></td>
<td>Southampton</td>
<td>Environmental</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VC3</td>
<td><em>V. cholerae</em></td>
<td>Southampton</td>
<td>Environmental</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VC4</td>
<td><em>V. cholerae</em></td>
<td>Southampton</td>
<td>Environmental</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VC5</td>
<td><em>V. cholerae</em></td>
<td>Southampton</td>
<td>Environmental</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VM499</td>
<td><em>V. mimicus</em></td>
<td>Dorset</td>
<td>Environmental</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VV11067</td>
<td><em>V. vulnificus</em></td>
<td>Dorset</td>
<td>Environmental</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>KIDBVV</td>
<td><em>V. vulnificus</em></td>
<td>Dorset</td>
<td>Environmental</td>
<td>+</td>
<td>+</td>
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<tr>
<td>LD365</td>
<td><em>Listonella damsela</em></td>
<td>Dorset</td>
<td>Environmental</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VB4</td>
<td><em>Vibrio spp.</em></td>
<td>Southampton</td>
<td>Environmental</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BL21</td>
<td><em>E. coli</em> (control)</td>
<td>Amersham</td>
<td>Unknown</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A – E correspond to all six loci described in Fig. 9.1 and Section 9.2
+/− Presence / absence of TTSS genes based on both PCR and dot blot analysis
*Identified with dot blot but not confirmed with PCR
Identified with PCR but not confirmed with dot blot

A - 209
Figure 9.6: Hybridisation signals of 55 isolates of *V. parahaemolyticus* with Probe A targeted to the *verD1* locus of TTSS1. The isolate corresponding to each dot is indicated. Rows correspond to the following: A and B clinical isolates; C and D environmental isolates from Dorset coast and Spain; E and F environmental isolates from Southampton, UK. White regions represent artefacts from photography of wet membrane.
**Figure 9.7: Hybridisation signals of 55 isolates of *V. parahaemolyticus* with Probes B and C targeted to the *vp1680* and *vscCJ* loci of TTSS1 respectively. The isolate corresponding to each dot is indicated. Rows correspond to the following: A and B clinical isolates; C and D environmental isolates from Dorset coast and Spain; E and F environmental isolates from Southampton, UK.
Figure 9.9: Hybridisation signals of 55 isolates of *V. parahaemolyticus* with Probe D targeted to the *vpa1321* locus downstream of the TTSS2 region of 'pathogenicity island' (VP-PA1). The isolate corresponding to each dot is indicated. Rows correspond to the following: A and B clinical isolates; C and D environmental isolates from Dorset coast and Spain; E and F; environmental isolates from Southampton, UK.

![Figure 9.9: Hybridisation signals of 55 isolates of *V. parahaemolyticus* with Probe D targeted to the *vpa1321* locus downstream of the TTSS2 region of 'pathogenicity island' (VP-PA1). The isolate corresponding to each dot is indicated. Rows correspond to the following: A and B clinical isolates; C and D environmental isolates from Dorset coast and Spain; E and F; environmental isolates from Southampton, UK.](image)

Figure 9.10: RAPD-PS1 clustering of TTSS2 positive isolates. Three clusters are shown in green, red and blue based on a 76% similarity value. Both TTSS1 and TTSS2 correspond to all three loci described. Isolates indicated with + were confirmed by PCR and dot blot whereas isolates marked with uc are unconfirmed by either PCR or dot blot. All isolates marked – are negative for both PCR and dot blot.

![Figure 9.10: RAPD-PS1 clustering of TTSS2 positive isolates. Three clusters are shown in green, red and blue based on a 76% similarity value. Both TTSS1 and TTSS2 correspond to all three loci described. Isolates indicated with + were confirmed by PCR and dot blot whereas isolates marked with uc are unconfirmed by either PCR or dot blot. All isolates marked – are negative for both PCR and dot blot.](image)
Table 9.4: Comparison of the presence of TTSS related genes A-F, virulence characteristics and phylogenetic RAPD Typing clusters in *V. parahaemolyticus*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Virulence Characteristics</th>
<th>TTSS1</th>
<th>TTSS2</th>
<th>RAPD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PSI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1902</td>
<td>NCIMB Reference</td>
<td>K-,tdh-,trh-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10884</td>
<td>NCTC Reference</td>
<td>K+,tdh+,trh+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>219</td>
<td>Clinical</td>
<td>K-,tdh-,trh-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>220</td>
<td>Clinical</td>
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1 Virulence characteristics
K: Kanagawa haemolysis and virulence genes (tdh, trh)

+/- Presence / absence of TTSS genes based on both PCR and dot blot analysis

\*: Identified with dot blot but not confirmed with PCR

\#: Identified with PCR but not confirmed with dot blot
Figure 9.11: Multiple alignments of the vcrD1 locus of TTSS1 in isolates of V. parahaemolyticus. RIMD2210633 is a sequenced clinical strain. Sequences 220A and VP416A are from clinical and environmental isolates respectively and 1902A is NCTC reference strain from which probes were developed (Corpet, 1988; Makino et al., 2003). Consensus regions are shown in red; blue and black show substitutions or deletions. Codon marked in green is valine (GTC) or isoleucine (ATC). Regions marked in dotted line represent quality control clipped regions. SNPs in TTSS1 are also present in vp1680 and vscC1 (Appendix III)
Figure 9.12: Multiple alignments of the *vpa1321* locus of TTSS2 in isolates of *V. parahaemolyticus*. RIMD2210633 is a sequenced clinical strain. Sequences 220D and VP416D are from clinical and environmental isolates respectively. (Corpet, 1988; Makino *et al.*, 2003). Consensus regions are shown in red, blue and black show substitutions or deletions. Regions marked in dotted line represent clipped regions. Nucleotide sequences of *vscC2* and *vcrD2* of TTSS2 are also conserved (Appendix III)
9.4 Discussion

Genome sequencing of *V. parahaemolyticus* (RIMD2210633) revealed TTSS regions, one with extensive homology to that described for *Yersinia* spp, and the other that had previously not been characterised before but was later found to show similarities to the TTSS2 found in non-O1 and non-O139 *V. choleae* (Dziejman *et al.*, 2005). Both TTSS1 and TTSS2 were shown to be functional and the fact that TTSS2 was only reported in Kanagawa positive (β-haemolytic) strains, suggest that it may be employed in the pathogenicity of *V. parahaemolyticus* (Ono *et al.*, 2006). The haemolytic reaction of thermostable direct haemolysin (TDH) is considered a marker of pathogenic strains (Sakurai *et al.*, 1973), although not all *tdh* bearing isolates exhibit this phenotype (Chapter 4; Nishibuchi *et al.*, 1989). Thus, the significance of finding *tdh* alone in environmental isolates remains unknown. Additionally, a small proportion of clinical isolates apparently lack both *tdh* and *trh* and the exact pathogenesis of *V. parahaemolyticus* has yet to be elucidated. The current work was carried out to assess the distribution of TTSS1 and TTSS2 regions in *V. parahaemolyticus* and may help to differentiate virulent and avirulent isolates of *V. parahaemolyticus*.

9.4.1 Gene targets on the TTSS system of *V. parahaemolyticus*

Three loci in TTSS1, two in TTSS2 and an additional locus recently identified just outside of the TTSS2 border comprising of the *V. parahaemolyticus* pathogenicity island (Vp-PA1) were targeted by PCR (Izutsu, *et al.*, 2008; Nishioka *et al.*, 2008). Two genes, *vcrDJ* and *vcsCJ* targeted on TTSS1 correspond to structural proteins with homologues found in *P. aeruginosa*, *S. typhimurium*, enteropathogenic *E.coli* (EPEC), *B. pseudomallei* as well as several other species (Hueck, 1998). Additionally, VP1680 described as a TTSS effector protein, to date only described in *V. parahaemolyticus*, was a third target for PCR in this study (Ono, Park *et al.*, 2006). The three loci were used to represent the entire TTSS1 region of approximately 40 kbp (Park *et al.*, 2004b). Similarly, the three genes *vpa1321*,

217
vscC2 and vcrD2 were selected to represent the entire TTSS2 region. The TTSS1 and TTSS2 are two distinct secretion systems. Cornelis and Van Gijsegem (2000) classified the TTSS of bacteria into three major groups: the Ysc-plus-Psc system, Salmonella pathogenicity-island ‘S-PAI-plus-Mxi/Spa’ system and a ‘S-PAI-plus-EPEC-EHEC’ system that constituted a system of Salmonella, enteropathogenic and enterohaemorrhagic E. coli genes. Whereas the TTSS1 system falls into the Ysc-plus-Psc system, the TTSS2 of V. parahaemolyticus is unlike any of the three described (Ono et al., 2006). Sequence alignments between vscC1 and vscC2 and between vcrD1 and vcrD2 of TTSS1 and TTSS2 in three experimental isolates and two sequenced strains revealed no significant homologies for the cross hybridisation of probes or PCR primers, thus were used as PCR and probe targets.

9.4.2 Detection of TTSS regions

Single and multiplex PCR targeted to the six loci described were generally found to be specific in amplifying the correct sized fragments in V. parahaemolyticus. Upon closer inspection, it seems unlikely that isolate Vib 1, (red arrow, Fig 9.4) showed an ambiguous sized band, as first thought. In this isolate only two TTSS1 sections were amplified with primers for vcrD1 and vscC1, showing what appeared to be an ambiguous sized fragment between 415 and 458 bp. However, dot blot analysis confirmed that all three loci were present and nucleotide sequence analysis would confirm the true identity of these bands.

Length polymorphisms in both virulence and housekeeping genes are widely reported in bacterial species. For example, PCR of the gene encoding toxin A (tcdA) in Clostridium difficile has shown length polymorphisms that enable some strains to be distinguished from others and a simple PCR to amplify capsule genes of Haemophilus influenzae can be used to discriminate between isolates (Rupnik et al., 1998; Falla., et al. 1994). However, in this study, it was necessary to confirm isolates lacking a PCR product by hybridisation.
analysis. A lack of a PCR product may be due to nucleotide mismatches, the lack of primer target sites in some isolates or even simply due to a reagent missing in the PCR reaction mix. Conversely, the presence of priming sites does not necessarily mean the correct region is being amplified in a strain especially if amplicon sizes vary from the expected. The finding of isolates that were positive for hybridisation and negative for PCR (and vice versa) illustrates the need for careful interpretation of PCR and hybridisation data.

To overcome the magnitude of large scale Southern blotting and to save time, a dot blot hybridisation system was employed. Six DIG-labelled probes were developed based on the PCR amplification products that generated two different signals. The type strain 1902 showed weak PCR signals for the TTSS2 regions (Fig. 9.4) and hence three additional probes targeting vpal321, vscC2 and vcrD2 were also developed from an O3:K6 serotype isolate 220. Despite isolate 1902 showing low PCR signals for TTSS regions, the probes developed from 1902 showed almost identical hybridisation profiles to those of probes developed from the template of isolate 220. However, the limitations of the dot blot analysis technique meant that a more detailed comparison of TTSS fragments between isolates was not possible. The finding of weakly positive signals together with the ‘halo effect’ present on some of the dots (most likely as a result of impurities in the DNA mixture deterring DNA cross-linking to membrane) indicate some of the drawbacks of this technique.

9.4.3 Distribution of TTSS1 genes in V. parahaemolyticus

Genes for TTSS1 were found in all 17 clinical isolates of V. parahaemolyticus in this study, including the two type strains; this is in agreement with previously published work (Makino et al., 2003; Ono et al., 2006; Park et al., 2004b). However, the presence of these genes in environmental isolates is much more variable. Out of 37 environmental isolates of V. parahaemolyticus used in the study, PCR and dot blot analysis revealed that four
isolates (VP345, VP361, VPEC1 and VPSC1) lacked all three of the TTSS1 loci examined, suggesting that the TTSS1 may not be an essential component for the survival of *V. parahaemolyticus* and may not be intrinsic to this species as suggested previously (Makino *et al.*, 2003; Ono *et al.*, 2006). An additional seven isolates (1024/5, 1023/3, Vib10, VP6, VP7, VP331 and VP491) were identified as possessing TTSS1 by dot blot hybridisation but not by PCR amplification, suggesting a lack of primer target sites. These findings support existing studies that have identified TTSS1 in all isolates of *V. parahaemolyticus*, both clinical and environmental (Makino *et al.*, 2003; Ono *et al.*, 2006; Park *et al.*, 2004b). A more extensive study with these environmental isolates using Southern hybridisation techniques and sequencing the entire TTSS region may help decipher structural variations in the TTSS1 region.

9.4.4 Distribution of TTSS2 genes in *V. parahaemolyticus*

Genes for TTSS2 were found both in clinical and environmental isolates of *V. parahaemolyticus*. These include isolates that showed presence or absence of the virulence genes *tdh* and *trh* regardless of whether they expressed the Kanagawa (β-haemolytic) phenotype or not (Table 9.4). This is contrary to earlier published information, suggesting that TTSS2 is only present in Kanagawa positive isolates (Makino *et al.*, 2003; Ono *et al.*, 2006; Park *et al.*, 2004b). However, the findings in this chapter are consistent with the latest report that clinical isolates that are not related to the pandemic clone and are not Kanagawa positive also exhibit TTSS2 (Meador *et al.*, 2007). Furthermore, as *vpa1321* lies approximately 10 kbp outside of the recently described boundary of the TTSS2 region within the *V. parahaemolyticus* pathogenicity island (Vp-PAI), it can also be proposed that all *V. parahaemolyticus* carrying a TTSS2 system reside on the Vp-PAI (Probe D, Fig. 9.9), which is typical of several pathogenic species with TTSSs (Hueck, 1998; Winstanley & Hart, 2001). This is further supported by the finding of the co-presence of the virulence gene *tdh* and TTSS2 in *V. parahaemolyticus* (Table 9.4) in most of the isolates. A copy of
the tdh gene (TDH-A) has been shown to lie close to the TTSS2 region approximately 4.3 kbp downstream of vpa1321 and, as expected, all but two isolates (VP7 and Vib9) that were positive for the vpa1321 locus in this study were also positive for tdh (Makino et al., 2003). The presence of TTSS2 together with the Vp-PAI and tdh all pertain to potentially pathogenic V. parahaemolyticus and thus routine analysis of all three regions rather than the tdh alone would better enable the monitoring of these strains.

Although TTSS systems are present in many Gram-negative species, to date only four other species have been characterised to possess two or more different TTSS in the same bacterium: S. typhimurium, Enterohemorrhagic E. coli O157: H7, Yersinia enterocolitica and B. psuedomallei (Hueck, 1998; Foultier et al., 2002; Stevens et al., 2002). In S. enterica, the first TTSS cluster (SPI-1) mediates enterocyte invasion while the second (SPI-2) is responsible for the intracellular survival within macrophages (Hensel et al., 1995). However, whilst the first TTSS of enterohaemorrhagic E. coli (LEE) is responsible for the attachment and formation of lesions in epithelial cells, the precise function of the second TTSS (LEE2) remains unknown (Ideses et al., 2005). Similarly, the presence of both TTSS gene clusters in all clinical isolates may suggest a requirement of both systems for the pathogenicity of V. parahaemolyticus. TTSS1 has been shown to secrete cytotoxic effector proteins from both the TTSS1 and TTSS2 regions, but the role of TTSS2 remains unclear (Ono et al., 2006). The finding of TTSS2 in at least 10 environmental isolates with both the presence and absence of tdh and trh genes have demonstrated that TTSS2 is not exclusive to clinical isolates as previously thought and these genes are present in other members of the Vibrio spp. (Table 9.3, Fig. 9.5).

9.4.5 Sequence diversity of TTSS1 regions

Sequence analysis of three isolates within the same six loci, together with the comparison with a further two sequenced strains has revealed that the TTSS2 regions, where present,
are more conserved than TTSS1. Single nucleotide polymorphisms (SNPs) were widely found in all three TTSS1 regions examined. These polymorphisms usually appeared on the third base of the codon and often did not alter the translated amino acid.

In bacterial pathogens, a variety of SNPs have been identified that may provide selective advantage during a course of an infection, epidemic spread or long term evolution of virulence (Sokurenko et al., 1999). For example, Boddicker and colleagues observed that two strains of *S. typhimurium* with comparable phenotypes demonstrated an eightfold difference in adhesion to human laryngeal epithelial (HEp2) monolayers. Two amino acid polymorphisms were identified in the high – binding *fimH* locus of the type 1 fimbriae and both changes were identified to two single nucleotide substitutions (Boddicker *et al.*, 2002).

Although some SNPs may be favourable and enhance pathogenicity, most cases of SNPs in both eukaryotic and prokaryotic systems are thought to result in gene knockouts (Weissman *et al.*, 2003). In the present study, valine was replaced with isoleucine in position 119 of *vcrD1* of TTSS1 in three pandemic O3:K6 strains (RIMD, 220 and VP416) upon comparison with the type strain. TTSS1 of isolate RIMD2210633 has been shown to be functional, and isolates 220 and VP416 exhibit strong β-haemolysis (Chapter 4). However, the large number of base substitutions identified in all three isolates raises the issue of whether the TTSS1 regions, more specifically the *vcrD1, vpl680* and *vscC1* genes, are functional and whether the presence of valine is responsible for a more virulent β-haemolytic phenotype. The finding of nucleotide deletions with resultant frameshift mutations in the *vpl680* locus in all three isolates sequenced raises additional support to question the functionality of this gene. A further comparison of these loci in a larger number of isolates may provide more conclusive information.
9.4.6 TTSS genes as epidemiological markers for virulent strains

To assess whether isolates possessing TTSS fell into distinct epidemiological clusters, RAPD-PS1 groupings were compared with TTSS positive isolates (Table 9.4). The finding that most of the environmental isolates possessing TTSS2 clustered in a hierarchical group that included four clinical isolates was of interest. Of the 10 environmental isolates that were confirmed for TTSS2 using both PCR and dot blot analysis, seven isolates fell into this unique cluster that was further divided into two sub-clusters (represented in red and blue, Fig. 9.10). Three isolates exhibited the presence of tdh; however, four isolates (1021/5, VP377, VP378 and VP939) in this group, as well as Vib3 from RAPD Type 24, were all negative for haemolysin genes tdh and trh. The close hierarchical clustering of these isolates negative for haemolysin genes and positive for TTSS2 warrants further investigation to determine their virulence status and to assess the feasibility of using TTSS2 as an additional virulence marker to tdh and trh. The finding of an environmental isolate (VP416) that is Kangawa positive (considered a strong indicator for virulent isolates) within this cluster may further suggest that other isolates may also be virulent in this group (Miyamoto et al., 1969).

9.4.7 Identification of TTSS1 and TTSS2 genes in other species

Since the identification of TTSS in *V. parahaemolyticus*, genome sequencing of a number of related vibrios have shown the presence of TTSS1 genes, including *V. harveyi*, *V. alginolyticus*, and *V. tubiashii* (Henke & Bassler, 2004; Park et al., 2004b). This work has additionally demonstrated these TTSS genes in *V. vulnificus* (VV11067), *V. anguillarum* (VanCM104) as well as confirming its presence in *V. cholerae* and *V. alginolyticus* (Dziejman, et al., 2005). Only the genes in non-O1, non-0139 *V. cholerae* have so far been shown to be homologous to the TTSS2 regions of *V. parahaemolyticus* (Dziejman, et al., 2005). Despite the finding of TTSS genes in other species, little is known about their activity. The ubiquitous nature of vibrios mean that a large array of virulence genes,
including some identified in clinical isolates of other species, may confer upon this organism a greater degree of virulence.

This chapter has described work to determine the presence of TTSS1 and TTSS2 genes amongst both clinical and environmental isolates, something which had previously been thought to be a feature of Kanagawa positive (beta-haemolytic) strains only. Although the presence of the TTSS1 has been shown to be responsible for translocating virulence determinants across the cytosol in *V. parahaemolyticus*, its role in pathogenicity with regards to the haemolysins TDH and TRH has not yet been established (Ono et al., 2006). It is likely that the presence of both the TTSS systems together with the variously described effector molecules result in pathogenicity. The finding of TTSS2 in a significant number of environmental isolates suggests that there may be more potentially pathogenic isolates in environmental reservoirs than currently thought.
Chapter 10

A COMPARISON AND EVALUATION OF METHODS USED FOR DIFFERENTIATING *VIBRIO PARAHAEOMOLYTICUS*

10.1 Introduction

In this present study, several phenotypic, serological and molecular techniques were applied in an attempt to assess and develop methods that may help differentiate pathogenic and non-pathogenic isolates of *V. parahaemolyticus*. Isolates were acquired from environmental, shellfish and clinical samples from several locations. Although etiological conclusions were hampered by the lack of information available on the isolates investigated, several techniques were successfully developed and applied. Phenotypic methods such as assessments of growth characteristics on general and selective media, β-haemolysis, O and K serotyping provided more discrimination amongst the isolates. The ITS1-PCR and tDNA-ILP methods measured localised variation within PCR amplifiable 16S-23S and tRNA spacer regions, whilst PFGE and RAPD provided a measure of interspecies variation throughout the genome. PCR methods, in general, were easy to execute and demonstrated varying degrees of diversity.

Previous chapters have discussed issues of typeability, reproducibility and discriminatory abilities of the individual typing system employed. This chapter aims as far as possible, to summarise, compare and contrast the properties and groupings obtained between different phenotypic and molecular typing systems by assessing measures of discrimination and concordance as applied to *V. parahaemolyticus* isolates throughout the study.
10.2 Materials and Methods

10.2.1 Phenotypic and growth characteristics of *V. parahaemolyticus*
Assessments of growth and haemolytic characteristics were summarised together with findings of O- and K- serotyping from Chapter 4. These methods are compared and evaluated.

10.2.2 Discrimination of molecular typing systems
The discriminatory power of the typing methods was estimated by the use of Simpson’s index of diversity, as defined by Hunter and Gaston (1988).

Discrimination index (DI):

\[ D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1) \]

Where \( N \) is the total number of strains used in the sample population, \( s \) is the total number of types described and \( n_j \) is the number of strains belonging to the \( j \)th type (Hunter & Gaston, 1988). For example, PFGE typing with the use of restriction enzyme *NotI* (Chapter 5) shows 30 Pulse Types (\( n_1 \) to \( n_{30} \)) where \( n_1 = 1, n_2 = 1, n_3 = 3, n_4 = 4, \) etc and \( N = 47 \). Calculations employing all 30 types give a discrimination index of 0.976. An index close to 0 indicates that little diversity is shown by the typing method whereas a DI approaching 1.0 reflects increasing diversity identified by the typing system. Discrimination indices were calculated for all five typing systems and fingerprint types.

10.2.3 Concordance of molecular typing systems
The concordance between the four typing systems was determined as previously using BioNumerics software (Version 5.1). Values from the similarity matrices of the two typing
systems under comparison were plotted on x and y axes. Each dot on the scatterplot represents corresponding similarity values for two isolates by any two of the typing methods. The graph therefore gives an indication of concordance between two typing techniques. The congruence, expressed as a percentage, was calculated in BioNumerics using the Pearson product-moment correlation coefficient (PMCC). The regression (r), based on the least square fitting of the regression line for the data points, was also calculated in BioNumerics and gives an indication of the reliability of the regression model. A first degree (linear) model with monotonous fit was employed. Second (r²) and third degree (r³) models were also compared for correlation of x and y data. A dendrogram showing the relationships of all the typing systems, including fingerprints from combined tD1, tD2 and tD3 data (tD-combined), and well as combined RAPD-PS1 and RAPD-PS2 (RAPD-combined) in the typing of V. parahaemolyticus was also produced.

10.3 Results

10.3.1 Growth of V. parahaemolyticus

Except for two environmental and one clinical isolate, growth on a selection of culture media (Chapter 4) in general, did not show any obvious signs of variability. Isolates VPEC1 and VPSC1 produced large amounts of exopolysaccharide on Wagatsuma agar and appeared clustered together in dendrogram analysis in all four of the molecular typing systems employed. The third isolate (6316) produced remarkably small colonies on TCBS agar, similar to V. mimicus, with unique fingerprint profiles throughout the course of the study. Based on Gram-staining, oxidase and basic morphological identification analysis as outlined in Chapter 4, all isolates (except 6316) appeared biochemically and morphologically indistinguishable with no apparent differences in growth characteristics. However, growth kinetics in reduced Na⁺ levels from 3.0% to 0.5% NaCl, significantly increased doubling times by 30% and supports recent evidence of increased fitness of environmental isolates at higher salinities (Martinez-Utarza et al., 2006). Environmental
isolates also appeared more tolerant to cold temperatures demonstrating increased culturability from cold storage.

10.3.2 Haemolytic characteristics

Out of 27 \textit{tdh} positive isolates used in the study, only 12 (44\%) isolates exhibited $\beta$-haemolysis; an attribute considered significant amongst clinical strains (Sakurai \textit{et al.}, 1973; Nishibuchi \textit{et al.}, 1989; Nishibuchi & Kaper, 1995). Of the 12 environmental isolates possessing the gene \textit{tdh}, only a single isolate, VP416, was haemolytic even though isolates were confirmed as TTSS2 positive in at least three other environmental isolates. The large proportion (29\%) of environmental isolates in the study possessing the \textit{tdh} gene is unusually high compared to typical levels of the \textit{tdh} gene (1 - 6 \%) found in environmental samples (Nishibuchi & Kaper, 1995) and is clearly the result of sampling bias and the acquisition of clinically significant isolates from culture collections. The suitability of the Kanagawa test as a means of differentiating \textit{tdh} positive strains is further questioned as three clinical isolates (2053, 5421 and E168143) possessing \textit{tdh} did not exhibit any haemolysis on Wagatsuma agar. This data suggests poor concordance between the gene \textit{tdh} and haemolysis. The difficulty in the availability of fresh rabbit (or human) blood, limits the use of KP reaction for the routine surveillance of TDH. Difficulty also arose in assessing the degrees of haemolytic activity as it was apparent that varying levels of haemolysis were present (section 4.3.4). An attempt to quantify haemolysis levels attributed to TDH using a spectrophotometer showed little success.

10.3.3 Serovar diversity of \textit{V. parahaemolyticus}

Out of a total of 63 isolates of \textit{V. parahaemolyticus}, 44 were serotyped into one of 20 O:K types (Chapter 4). Serotyping had a good discriminatory power (0.937) where all O antigens were typeable with one of the 13 O-antisera commercially available. However, not all K-antigens were typeable and were designated KUT (Table 10.2 & 10.3). Of the 71
K-antigens currently available for *V. parahaemolyticus*, the isolates tested fell into one of 14 K-types that included KUT. Nine clinical isolates in the study were of the O:K6 serotype. A further two O3 serotypes (isolates 225 & 226) were K-untypable but were positive for all four ITS1 and RAPD markers that are found present in the pandemic clones (Table 10.2). Within clinical isolates, an additional six serovars were present reflecting a diverse O:K population of disease causing types. A number of studies have shown a wide variety of serovars responsible for human disease (Nair *et al.*, 1985; 2007). Serotypes amongst environmental isolates varied widely, with the predominant O1-type, present in 55% of environmental isolates. Other than the presence of O3:K6 isolates amongst clinical isolates, there appeared to be no other apparent serovar association in clinical and environmental isolates or their respective markers.

As well as serotyping being a discriminative method, it also forms an easily reproducible technique due to the standardised reagents available in the serotyping kit. However, serotyping proved to be labour intensive and expensive. The availability of antisera for *V. parahaemolyticus* O and K antigens is difficult, with only a few distributors worldwide (Kaysner & DePaola, 2004). With the rise of pandemic clones of serotypes O3:K6, O1:KUT, O1:K25 and O4:K68 as the dominant serovars, the present limited study highlights the investigation of the RAPD markers (Bands X and Y) for the potential identification of pandemic isolates.

10.3.4 Comparative analysis of pulsed-field gel electrophoresis

In chapter five, *NotI* was used to generate restriction profiles of *V. parahaemolyticus* with PFGE. Of the 59 isolates investigated using the methods described, a total of 47 isolates were used for dendrogram construction which included isolates that exhibited various degrees of DNA degradation. PFGE analysis, together with serotype variation, confirmed a heterogeneous population of *V. parahaemolyticus* under investigation with 30 Pulse Types
and with the largest clusters (PT 04 & PT 18) containing only 4 isolates each. Despite the untypeability of approximately 20% of the isolates, PFGE showed the highest discrimination index (0.976) of all typing systems (Table 10.1). Apart from environmental isolates from Southampton (Vib1 to Vib3 & Vib7 to Vib10) that appear to be clonally related or even the same, isolates from the Dorset coast generally appeared untypeable; those that were typeable could not be assigned into clusters based on the location or the host source.

In general, PFGE typing showed low correspondence with all the other typing systems (Figs. 10.1 – 10.3) with congruence values ranging from 10.5% (with tD3) to 21.6% (with RAPD-combined). A comparison of PFGE with RAPD-PS1 and RAPD-PS2 showed that RAPD-PS2 was more congruent to PFGE than RAPD-PS1, with the composite RAPD PS1 & PS2 data appearing more congruent (Figs. 10.1 & 10.2).

Isolates that appeared grouped in pairs in PFGE such as VPSC1 and VPSC1 or 6134 and VP416 were also identified grouped together in RAPD-PS1 and RAPD-PS2 (Chapters 5 & 8: Figs. 5.11, 8.10 & 8.12. Clonally related O3:K6 isolates were also generally grouped together in all three fingerprint types to further support the reliability of these groupings. However, despite some similar groupings, there appeared general discordance. Firstly, isolate E154482 appeared closely related to two clinical isolates from the UK, E155855 and E168482 in RAPD-PS1 whereas PFGE and RAPD-PS2 clearly differentiated isolate E154482 from the two pandemic O3:K6 isolates. Furthermore, a few groups were attributed to different lineages by dendrogram analysis of different fingerprint types. For example, isolates Vib1 - Vib3 and Vib5 - Vib10 that were found grouped together in most of the fingerprint types, appeared in the same hierarchical branch as pandemic isolates in RAPD-PS1. However, the same isolates appeared to branch off into different groups in PFGE. Such differences may explain such low congruence values.
10.3.5 16S-23S intergenic spacer (ITS1) based typing

Independent experiments showed that the ITS1-PCR typing method developed was reproducible with respect to the two main amplicons of approximately 390 and 635 bps. Other than isolate 6316, these two bands distinguished *V. parahaemolyticus* from other species (Chapter 6). Up to a total of eight bands within 390 to 810 bps were present with considerable variability of intensity. At 93% similarity, 13 groups were identified that included a single large group accounting for 56% of the isolates. An overall discrimination index of 0.665 and 100% typeability amongst the 56 isolates (Table 10.1) was shown with ITS1-PCR. This method was shown to be a simple technique to assess intra-species relationships but a thorough investigation with accurate band sizing techniques is required to determine whether clinical or environmental isolates possess unique banding patterns.

Due to the unavailability of a complete set of PFGE data, a visual comparison of groups obtained by RAPD-PS1 and ITS1-PCR was made. Clonal groups (Table 10.2) in ITS1-PCR could not be as easily distinguished as RAPD-PS1. Based on ITS1-PCR, these clinical isolates appeared indistinguishable from several environmental isolates from Spain and Southampton, although at sufficiently high similarily values (>95%), clonal isolates appeared to be clustered together (Chapter 6: Fig. 6.11). Several other groups such as VPEC1 and VPSC1 and Vib1 to Vib10 were also found clustered together in both RAPD-PS1 and ITS1-PCR. In addition to these concordant groups, a larger and significant number of discordant types were also identified. A visual representation of the data by means of a scatterplot demonstrated low concordance both with RAPD-PS1 and RAPD-PS2 with values of 25% and 32% respectively (Fig. 10.4). A comparison with PFGE, even though a significant number of isolates were untypeable, similarly showed low correspondence (Fig. 10.2: B). It appears that these comparisons do not follow a linear model and using a quadratic or higher model (r² or r³ respectively) demonstrated higher regression values (Table 10.3). However, a comparison of ITS1-PCR with tDNA-ILP showed a higher...
degree of concordance of between 35.5 and 51.8%. Of the three tDNA-PCR typing systems (tD1, tD2 & tD3), tD3 showed at least 16% less concordance of ITS1-PCR than tD1 or tD2 (Fig. 10.1). In general, ITS1-PCR did not differentiate *V. parahaemolyticus* isolates as well as PFGE or RAPD, but provided general information on the isolates available. Almost all of the clonally related (Table 10.2) types were found clustered together in the largest group (ITS1-C) and thereby these isolates were not differentiated by ITS1-PCR. Vib1 to Vib3 and Vib5 to Vib8 and Vib10 that clustered together based on ITS1 polymorphisms, could not be grouped together with any of the tDNA-ILP fingerprint types.

**10.3.6 tDNA-ILP based typing**

The use of three sets of consensus tRNA gene primers provided a set of data representative of tDNA ILP typing of *V. parahaemolyticus*. Discrimination indices were 0.724, 0.568 and 0.703 (for fingerprint types tD1, tD2 and tD3 respectively) with 100% typeability in all three fingerprint types. Up to eight amplicons were identified in PCR analyses with a good degree of reproducibility of the main amplicons in repeat tests. Only a single isolate (VP10884) failed to amplify one of the four main amplicon of approximately 740 bp found amongst the 6 isolates tested. As with ITS1-PCR, variation in reproducibility lay mainly in the amplification of weak bands in tDNA-PCR. A comparison of fingerprint types tD1, tD2 and tD3 with the combined data of all three tDNA-PCR experiments showed that tD1 was the most differentiating of the three sets of primers and therefore used for comparative purposes (Fig. 10.1). A lower discrimination index was obtained for tD2 even though isolates in tD2 were grouped at a higher similarity value of 95% instead of 90% as with tD1 further suggesting primers for tD2 was the least discriminative of the three sets of tDNA primers.
Fingerprint type tDNA-ILP (tD1) was compared to RAPD-PS1. A fair degree of concordance was demonstrated between the two fingerprint types (Congruence = 47%, r = 56.7%, Fig. 10.5:A). For example, isolates VP1 to VP10 that formed two groups in RAPD-PS1 was also identified within a subset of the largest group of tD1 (tD1 cluster A). Similarly, most isolates of the pandemic clonal group (Table 10.2) appeared clustered together in the comparison (Fig. 7.6, cluster tD1-C). Two further, smaller, groups consisting of isolates VP331, VP345, VP361, VPEC1 and VPSC1) were also grouped in both fingerprint types. It is not known to what extent this could be attributed to isolates placed on the same gel and therefore this needs to be addressed in further studies. Most other environmental isolates appearing heterogeneous in more sensitive methods such as RAPD and PFGE were found clustered together and sometimes indistinguishable based on tDNA-ILPs. Likewise, a comparison of tD1 with RAPD-PS2 demonstrated some degree of concordance (Congruence = 56.6%, r = 62.1%).

10.3.7 Randomly amplified polymorphic DNA

RAPD was shown to be the second most discriminative method after PFGE with discrimination indices of 0.965 and 0.959 for the two RAPD fingerprint types, PS1 and PS2 respectively. The two-primer RAPD methods developed were almost as discriminative as PFGE (DI = 0.976) but unlike PFGE, did not suffer from issues of untypeability and were thus used as a method to which other typing systems were compared. RAPD identified 30 and 24 RAPD types at ≥ 90% similarity with PS1 and PS2 respectively, with good correspondence between the two RAPD types (Section 8.3.6 & Fig. 10.6). However, despite this discriminatory ability and congruence between two sets of RAPD primers, concordance with PFGE was low (Fig 10.1). For example, Vib6 that appeared similar to clinical isolate E154482 by PFGE analysis was identified in different groups by both RAPD methods. Examination of ITS1-PCR and tDNA-ILPs found little correspondence between isolates Vib6 and E154482. Thus in this case, similarities observed by PFGE
could not be verified by other typing clusters. In addition, the RAPD method appeared to be the least reproducible in comparison with ITS1-PCR and tDNA-ILP. Nonetheless, the RAPD method identified two possible markers that corresponded well with the pandemic group specific (GS) PCR (Table 10.2). Further investigation is required to confirm whether such bands are unique to pathogenic isolates only.

10.3.8 Evaluation of the TTSS2 in *V. parahaemolyticus*

Based on the data presented in Chapter 9, isolates confirmed as possessing the second type three secretion system (TTSS2) were those that gave positive signals for both PCR and DNA probe hybridisation for at least two of the three loci examined. The groupings and comparisons of TTSS2 positive isolates are shown in Table 10.2. For comparative purposes, genotypes and characteristics of TTSS2 positive and TTSS2 negative isolates are presented in Tables 10.2 and 10.3 respectively. It became immediately apparent that all clinical isolates and a significant number of environmental isolates possessed a TTSS2.
Table 10.1: Discrimination of *V. parahaemolyticus* typing methods used in this study

<table>
<thead>
<tr>
<th>Method (Chapter)</th>
<th>Fingerprint Type</th>
<th>No. of Types&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Size (%) of largest type</th>
<th>DI&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Similarity of types&lt;sup&gt;4&lt;/sup&gt;</th>
<th>% Typeability&lt;sup&gt;5&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotyping&lt;sup&gt;1&lt;/sup&gt; (4)</td>
<td>n/a</td>
<td>20</td>
<td>19</td>
<td>0.937</td>
<td>n/a</td>
<td>70</td>
</tr>
<tr>
<td>PFGE (5)</td>
<td>Not1</td>
<td>30</td>
<td>4</td>
<td>0.976</td>
<td>≥ 88%</td>
<td>80</td>
</tr>
<tr>
<td>ITS1-PCR (6)</td>
<td>ITS1</td>
<td>13</td>
<td>57</td>
<td>0.665</td>
<td>≥ 93%</td>
<td>100</td>
</tr>
<tr>
<td>tDNA-ILP (7):</td>
<td>tD1</td>
<td>7</td>
<td>45</td>
<td>0.725</td>
<td>≥ 90%</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>tD2</td>
<td>7</td>
<td>64</td>
<td>0.568</td>
<td>≥ 95%</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>tD3</td>
<td>9</td>
<td>46</td>
<td>0.703</td>
<td>≥ 95%</td>
<td>100</td>
</tr>
<tr>
<td>RAPD (8):</td>
<td>PS1</td>
<td>30</td>
<td>13</td>
<td>0.965</td>
<td>≥ 90%</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>PS2</td>
<td>24</td>
<td>11</td>
<td>0.959</td>
<td>≥ 90%</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>1</sup> Serotyping is character type data; Chapter 4: Table 4.3. Typeability refers to untypeable K-antigen only. All O-antigen typing, where performed, were typeable.

<sup>2</sup> Includes types assigned at ≥ 88% similarity.

<sup>3</sup> Discrimination Index based on Hunter & Gaston (1988); a value closer to 1.0 suggests a more discriminate typing system.

<sup>4</sup> Based on dendrogram

<sup>5</sup> Typeability was scored as a percentage of the typing system to assign a type to each isolate (Struelens, 1996)
Figure 10.1: Summary of concordance for all molecular typing systems. Matrix shows percentage congruence values as calculated with the PMCC in BioNumerics. Composite data for all three tDNA-PCR and both RAPD experiments are marked as tD-combined and RAPD-combined respectively. Dendrogram shows a measure of the relatedness of the molecular typing systems based for the typing of V. parahaemolyticus.
Figure 10.2: Concordance of stratification by PFGE and RAPD for *V. parahaemolyticus* isolates. Each dot represents corresponding similarity values for two isolates obtained by PFGE and RAPD-PS1 typing (A) and PFGE and RAPD-PS2 typing (B). Concordance as calculated with the PMCC is shown as C and regression by r.
Figure 10.3: Concordance of stratification by PFGE and tDNA-ILP typing (A), and PFGE and ITS1 typing (B) for *V. parahaemolyticus* isolates. Each dot represents corresponding similarity values for two isolates obtained by PFGE, tDNA-PCR (tD1) and ITS1-PCR. Concordance as calculated with the PMCC is shown as C and regression by r.
Figure 10.4: Concordance of stratification by ITS1-PCR and RAPD for *V. parahaemolyticus* isolates. Each dot represents corresponding similarity values for two isolates obtained by ITS1-PCR and RAPD-PS1 typing (A), and ITS1-PCR and RAPD-PS2 typing (B). Concordance as calculated with the PMCC is shown as C and regression by r.
Figure 10.5: Concordance of stratification by tDNA-ILP and RAPD for *V. parahaemolyticus* isolates. Each dot represents corresponding similarity values for two isolates obtained by tDNA-PCR and RAPD-PS1 typing (A), and tDNA-PCR (tD1) and RAPD-PS2 typing (B). Concordance as calculated with the PMCC is shown as C and regression by r.
Figure 10.6: Concordance of stratification by tDNA-ILP (tDI) and ITS1-PCR for *V. parahaemolyticus* isolates. Each dot represents corresponding similarity values for two isolates obtained by tDNA-PCR and ITS1-PCR typing. Concordance as calculated with the PMCC is shown as C and regression by r.

\[ C = 46.5\% \quad r = 62.9\% \]
Table 10.2: Comparison of *V. parahaemolyticus* TTSS2 isolates and typing groups to distinguish virulent and potentially virulent strains. All isolates are confirmed TTSS2 positive both by PCR and dot blot hybridisation.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Serotype</th>
<th>PCR&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Typing groups&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>tdh</td>
<td>trh</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>220*</td>
<td>O3:K6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>222*</td>
<td>O3:K6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>223*</td>
<td>O3:K6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>227*</td>
<td>O3:K6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E155855*</td>
<td>O3:K6</td>
<td>+</td>
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<td>E168143*</td>
<td>O3:K6</td>
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<tr>
<td>2053*</td>
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<tr>
<td>5421*</td>
<td>O3:K6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>221*</td>
<td>O1:KUT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>225*</td>
<td>O3:KUT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>226*</td>
<td>O3:KUT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>219</td>
<td>O6:KUT</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>224</td>
<td>O3:K6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>228</td>
<td>O11:KUT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2436</td>
<td>O6:K18</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6134</td>
<td>O4:K12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E154482</td>
<td>O1:K1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Type Strains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1902</td>
<td>O1:K1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VP10884</td>
<td>O3:K4</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Environmental</td>
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<td></td>
</tr>
<tr>
<td>1021/5</td>
<td>O2:KUT</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Vib3</td>
<td>O1:K32</td>
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<td>-</td>
</tr>
<tr>
<td>Vib7</td>
<td>ND</td>
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<td>-</td>
</tr>
<tr>
<td>VP2</td>
<td>ND</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VP10</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
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<td>VP331</td>
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<td>O4:KUT</td>
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<td>O6:K18</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>VP939</td>
<td>O4:K18</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>1</sup> Wagley et al., (2008)

*tdh*: thermostable direct haemolysin (TDH) gene

*trh*: TDH related haemolysin gene

*GS*: Group Specific PCR for pandemic types (Matsumoto et al., 2000)

<sup>2</sup> Kanagawa phenomenon – β-haemolytic isolates

*Groups based on ≥88% similarity. PF: Pulsed-field gel electrophoresis with NotI, ITS: ITS1-PCR, tD1-tD3: tDNA-PCR with three primer pairs, RAPD-PS1 and RAPD-PS2 types indicated with * correspond to distinct amplicons (Band X and Band Y) as possible markers of pandemic strains in RAPD-PS1 and RAPD-PS2 respectively.

ND: Not determined

UT: Untypeable

Clonal (*) and clonally related isolates (•) based on GS-PCR, PFGE, RAPD, ITS and tDNA PCR.
Table 10.3: Comparison of TTSS2 unconfirmed (UC) or TTSS2 confirmed negative (-) isolates of *V. parahaemolyticus* to potentially distinguish avirulent strains. All isolates are environmental.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Serotype</th>
<th>PCR1</th>
<th>Typing groups2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>tdh</em></td>
<td><em>trh</em></td>
</tr>
<tr>
<td>TTSS2 (UC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H023/1</td>
<td>O1:K32</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H023/9</td>
<td>O1:K1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vib1</td>
<td>O1:K32</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vib3</td>
<td>O1:K32</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vib6</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>Vib8</td>
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<td>Vib10</td>
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<tr>
<td>VP1</td>
<td>O1:K33</td>
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<tr>
<td>VP3</td>
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<td>VP8</td>
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<tr>
<td>VP346</td>
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<tr>
<td>VP7</td>
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<td>-</td>
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<tr>
<td>VP361</td>
<td>O1:KUT</td>
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</tr>
<tr>
<td>VP491</td>
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<td>-</td>
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<td>VP5C1</td>
<td>O8:KUT</td>
<td>-</td>
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1 Wagley et al., (2008)

tdh: thermostable direct haemolysin (TDH) gene

trh: TDH related haemolysin gene

GS: Group Specific PCR for pandemic types (Matsumoto et al., 2000)

2 Kanagawa phenomenon – β-haemolytic isolates

3 Groups based on ≥88% similarity in typing systems. PF: Pulsed-field gel electrophoresis with NorI, ITS: ITS1-PCR, tDI-tD3 corresponds to tDNA-PCR with three primer pairs.

ND: Not determined

UT: Untypeable
Table 10.4: Analysis of regression in comparison of typing systems. Regression models $r_1$, $r_2$ and $r_3$ correspond to first, second and third degree regression models respectively. Values are as calculated from BioNumerics software using the method of least squares. Figures 10.2 to 10.6 show a $r_1$ linear regression line whereas in some instances $r_2$ and $r_3$ curves provide better data fit. Increasing regression values were obtained with higher regression models in comparisons where localised variation (i.e. typing systems tDNA-PCR or ITS1-PCR) was compared with high discriminatory genome-wide variation (i.e. PFGE or RAPD).

<table>
<thead>
<tr>
<th>Regression model</th>
<th>Typing comparisons</th>
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<tr>
<td></td>
<td>PFGE</td>
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<td></td>
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<tr>
<td>$r_1$</td>
<td>34.4</td>
</tr>
<tr>
<td>$r_2$</td>
<td>40.8</td>
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<tr>
<td>$r_3$</td>
<td>40.9</td>
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10.4 Discussion

Subtyping or isolate classification can be accomplished by using a number of different techniques. These methods must meet several criteria in order to be useful. A comparison of different typing methods used in this study has outlined various degrees of typeability, reproducibility and discriminatory powers, together with an evaluation of the ease of use of a typing system. This chapter aimed to compare and evaluate the groupings obtained to help establish relationships and assess the reliability of the methods and the relatedness inferred by different characterisation and typing systems.

10.4.1 Phenotypic methods for isolate differentiation

The TDH phenotype could not be attributed to a significantly large proportion of \textit{tdh} gene possessing isolates. The haemolysin TDH is known to be a pore forming toxin that causes cytotoxicity in a Ca\textsuperscript{2+} independent pathway (Honda \textit{et al.}, 1992; Tang \textit{et al.}, 1995; Takahashi \textit{et al.}, 2006). However, the precise mechanisms of its mode of action still remain unknown. The present data show a greater proportion of environmental isolates possessing the gene \textit{tdh} than clinical isolates not exhibiting haemolysis. It is not known whether the gene \textit{tdh} is switched off and/or present in an inactive form amongst environmental isolates. The conserved nature of \textit{tdh} (Nishibuchi \& Kaper, 1995) may suggest, however, that the copy is functional. The significance of finding \textit{tdh} in environmental isolates therefore remains unclear. Of interest would be to assess the actual numbers of environmental isolates harbouring \textit{tdh} genes to exhibit haemolysis. Non-haemolytic clinical isolates possessing the gene \textit{tdh} indicate that passage in the human host may not be responsible for the activation of \textit{tdh} genes.

As far as the author is aware, antisera for \textit{V. parahaemolyticus} O and K antigens is only produced by Denka-Seiken in Japan with a few distributors worldwide (Kaysner \& DePaola, 2004). The costs and labour associated with serotyping, together with the
untypability as shown in the present study, and by others (Nair et al., 1985) suggest that serotyping of a large number of environmental isolates may not a feasible option.

10.4.2 Typing methods of *V. parahaemolyticus*

PFGE was performed so that it could be used as a “benchmark” typing method to which additional typing systems, which were simpler to perform, could be compared. Despite the recent trend towards nucleotide sequencing typing methods, to date, PFGE remains the most widely adopted method for typing nosocomial, community acquired and foodborne pathogens (Tenover et al., 1997; Hyytia-Trees et al., 2007). Due to the large degree of untypeability of *V. parahaemolyticus* by PFGE (and thus the unavailability of a complete set of PFGE data) for all 56 isolates of *V. parahaemolyticus*, an initial comparison of PFGE with both RAPD-PS1 and RAPD-PS2 was undertaken.

Struelens (1996) suggested that a sufficiently discriminate typing system should have a discrimination index of at least 0.950 and therefore both PS1 and PS2 have shown to be adequate for subtyping of *V. parahaemolyticus*. In a previous comparative study, using a single primer RAPD method, Wong et al., (2001) identified several typing systems with RAPD as the lowest discrimination index (0.840). The workers found ERIC (DI = 0.980) and REP-PCR (DI = 0.97) to be more discriminatory than PFGE (DI = 0.960) based on 40 strains typed (Wong et al., 2001). Whilst the present PFGE data (DI = 0.976) support these findings, the two-primer RAPD method (DI = 0.959 - 0.961) has been shown to be a more discriminate method and presents an enhanced typing system achievable without significant addition to costs or labour times. However, based on the present study, low concordance of PFGE was shown with all the typing systems. PFGE suffered from high degrees of DNA degradation where a significant proportion of isolates were not typeable. Of those that appeared typeable, a further subset (such as isolate 226 and VP6 – VP9) showed distinctly weak bands. It is likely that some bands could not be identified amongst
the DNA smears. Furthermore, based on scatterplots and regression analysis it seems likely that some of the comparisons could better be associated with quadratic or higher models (i.e. $r_2$ or $r_3$) rather than a linear relationship. A comparison between first degree (linear) and non-linear models showed better data fit with non-linear models in some instances. This seemed more apparent when comparisons were made between high and low discriminatory typing systems. BioNumerics employed a Pearson product-moment correlation coefficient to assess the linear relationships between two comparative typing methods. As some models don’t appear linear, it is possible that small changes apparent in ITS1 and tDNA spacer regions identified from typing experiments could be indicative of greater or lesser genome variation as measured by RAPD or PFGE.

The use of RAPD for typing *V. parahaemolyticus* has been suggested by others, above all on the grounds of the ease of use before more labour intensive approaches such as MLST and VNTR analyses are performed (Giske *et al.*, 2006). However, inter-laboratory reproducibility of this method is a widespread concern and has been discussed earlier (section 8.4.6). Despite this, the data presented in this thesis show some degree of reproducibility in independent experiments and this may be partially due to the use of standardised reagents such as RAPD analysis beads (Amersham Biosciences). However, in general, levels of reproducibility did not appear to be adequate and further work is required. Based on the preliminary studies described in this report, the use of ITS1-PCR and tDNA-ILP offer less discriminative alternatives to RAPD. The work was carried out with standard, easily available laboratory reagents and may prove to be more robust than RAPD in inter-laboratory assessments. For example, changing annealing temperatures did not significantly affect banding patterns in ITS1-PCR in the present study and therefore variation arising as a result of PCR amplification at high annealing temperatures could be more attributed to mutations in the primer binding sites. This is in contrast to the low annealing temperatures for RAPD that would result in more non-specific primer
hybridisation to DNA template (Bart-Delabesse, et al., 2001). Therefore, ITS1-PCR and tDNA-ILP warrant further analysis on larger sample numbers.

The use of more sensitive typing methods such as PFGE and RAPD may not always be appropriate. For example, environmental populations of *V. parahaemolyticus*, as shown in this study, and by others, are genotypically diverse (Nair et al., 1985). Both clinical and environmental isolates were identified as single members in 18 and 20 of their own types in RAPD-PS1 and PFGE respectively. Therefore, methods such as ITS1-PCR or tDNA-ILP may be satisfactorily used as a first step towards strain confirmation and differentiation. The present work has collectively demonstrated that the less discriminate methods grouped distantly related *V. parahaemolyticus* isolates and provides useful low resolution typing information. Combining data from multiple experiments, when high similarities are obtained (i.e. tD-combined) can provide greater differentiation than single primer pairs alone but in the present case, combined data appears to group together isolates from the same gels more than single gels alone thereby exemplifying the need for random isolate distribution on a gel and careful data analysis.

As described in section 6.4.5, a number of tRNA genes can be found within ITS1 regions of *V. parahaemolyticus* (Makino et al., 2003). Whereas ITS1-PCR provided a measure of spatial heterogeneity between conserved ribosomal genes, the genome wide distribution of tRNA genes provided a method in which more of the genome was subject to analysis. This may help to explain the discrepancy in concordance of 46.5% (linear regression = 62.6% fit) between ITS1 and tDNA (tD1) ILP typing. It is not known if the tRNA gene primers used were specific for particular tDNA regions encoding for specific tRNA classes or whether such inferences could be made. However, based on the amplicon sizes it would appear that there were limitations posed by PCR amplification conditions. For example, the largest amplicon from ITS1-PCR (810 bp) was expected in three copies ((Makino et al., 248
2003; Gonzalez-Escalona et al., 2005b; 2006a) and hence to result in an intense band. Clearly this was not the case observed as amplicons above 700 bp appeared faint (Chapter 6). Similarly, tDNA-ILP bands (Chapter 7) above 600 bp appeared faint. In both cases, there were significant amounts of background PCR noise that would need to be addressed in further studies employing these methods.

10.4.3 General reliability of clustering patterns

It was apparent that no two typing systems used in the present study gave a high concordance and there remain questions on the reliability of the data obtained. The discordance of PFGE with other typing systems highlights the need for the use of an additional restriction enzyme for PFGE analysis. As both RAPD-PS1 and RAPD-PS2 appear congruent to each other and to some degree with ITS1-PCR and tDNA-PCR typing, it is likely that the current PFGE data needs further reassessment. For example, similar banding isolates such as VP6 - VP9 appeared distant on the PFGE dendrogram that may suggest sub-optimal BioNumerics band tolerance settings. As mentioned earlier, this could also arise if faint bands were not selected in the analysis. A similar explanation of isolates that appeared to share the same apparent fingerprint pattern yet were clustered differently in ITS1, tDNA and RAPD dendrograms can be provided. It is also worthy of note that analysis of PFGE was performed using the Dice, band-based method, whereas other typing systems employed a curve based method. Analysis of the concordance tests were limited by the reliability of the data originally obtained and may further explain low congruence values in the comparisons. Discordant results between RAPD and PFGE have been reported before. In a recent external quality assessment collaborative study with a network of laboratories, RAPD was shown to be 47% concordant to PFGE with Staphylococcus aureus isolates (Deplano et al., 2006). In another study, RAPD demonstrated only 26.5% concordance with other typing techniques such as RFLP (Bart-Delabesse, et al., 2001). Despite such discrepancies, RAPD continues to be widely employed in typing systems.
It was found with PFGE that isolate 6316 showed more similarity to Vib6 and E154482 than any of the other isolates, whereas both ITS1-PCR and tDNA-ILP typing clearly discriminated 6316. With a large number of bands (>15) and a few common bands, this isolate did not show as much variability by PFGE for it to be classified as such. As mentioned earlier, isolate 6316 showed unique growth characteristics similar to that of *V. mimicus*. The placement of this isolate in the PFGE dendrogram thus seems inappropriate and can be used as a measure to further illustrate the genetic diversity of *V. parahaemolyticus* isolates under study. Therefore ITS1-PCR or tDNA-ILP typing provided further evidence that isolate 6316 may not be closely related to *V. parahaemolyticus* or may even be a different species altogether.

Inconsistencies in groupings obtained in the typing systems may also partially be attributed to the method used for dendrogram analysis. In the ‘curve based’ analysis methods used, it was found that the presence of much darker bands gave unreliable clusters where faint bands appeared ignored. This has been identified by others as an inherent problem of curve based analysis methods (Werner *et al.*, 2003; Rasschaert *et al.*, 2005) and based on present data seems more of a concern when there are few bands generated in the typing system.

**10.4.4 Differentiation of clonally related *V. parahaemolyticus***

There are an increasing number of reports of the prevalence and dominance of clonally related *V. parahaemolyticus* in disease outbreaks worldwide (Chiou *et al.*, 2000; Chowdhury *et al.*, 2004b; Ansaruzzaman *et al.*, 2005; Rizvi *et al.*, 2006; Serichantalergs, *et al.*, 2007). To date, these isolates generally have been shown to fall into one of four serotypes: O3:K6, O1:KUT, O1:K25, and O4:K68 (Chowdhury *et al.*, 2004a), and appear indistinguishable even with PFGE methods, contrary to the belief that isolates with highly related PFGE patterns shared the same phenotype (Hall *et al.*, 1996). Only a small number
of isolates appeared clonal in the present study (four isolates of PFGE type 18; Fig. 5.11). The remaining seven isolates were categorised as clonally related (Table 10.9) due to incomplete isolate histories. Based on this data alone and the limited number of isolates, it is difficult to postulate the extent to which PFGE has differentiated clonally related isolates in this study.

Recent developments in MLST for *V. parahaemolyticus* provide more appropriate means to assess diversity amongst isolates indistinguishable by PFGE or other means (Chowdhury *et al.*, 2004b; Gonzalez-Escalona *et al.*, 2008). Amongst a total of 37 clinical and 63 environmental isolates, three major clonal complexes and 62 sequence types were identified amongst seven house-keeping genes (Gonzalez-Escalona *et al.*, 2008). The study showed the same MLST sequence type (ST) in four different serotypes and thus further supports the non-correspondence between serotype and molecular based typing systems.

### 10.4.5 Conclusion

To date, there appear no published studies of the typing of *V. parahaemolyticus* by ITS1-PCR or by tDNA-ILPs. This study is the first to compare these methods with RAPD and PFGE typing. The initial findings in this work suggest both ITS1-PCR and tDNA-ILP methods are applicable for routine analysis with a two-primer RAPD method far easier to execute than PFGE, but gave almost equivalent discrimination. The different molecular typing systems generated different groupings and lineages, and the results, in general showed various degrees of concordance. This was expected with different typing systems as each typing method targets different loci and in some cases not enough loci may be targeted to result in a true reflection of genome diversity. For example, it is unlikely that ITS1-PCR typing with species with a low ribosomal RNA copy number would give sufficient variation of length polymorphisms to be able to distinguish between isolates. Serotype data and the PCR identification of virulence genes in shellfish and seafood
samples, although useful adjuncts to characterise pathogenic strains, do not necessarily provide useful information about the virulent status of the organism. The identification of TTSS2 in all clinical isolates and a significant proportion of environmental ones, may be a more useful a marker for virulent *V. parahaemolyticus*. 
Chapter 11

GENERAL DISCUSSION AND CONCLUSIONS

11.1 Introduction

Members of the genus *Vibrio* are ubiquitous in the marine and estuarine environment. Accurate and reliable methods for the identification and differentiation of pathogenic vibrios are thus essential if epidemiological studies are to be successful. This study employed several simple methods that could easily be adopted to assess diversity amongst populations of *Vibrio* spp. commonly found in shellfish and other seafood samples. Additionally, as conventional approaches to the identification and confirmation of *V. parahaemolyticus* are lengthy and labour intensive, some of the methods described in this work may help to identify *V. parahaemolyticus* and ease the epidemiological assessment of large samples. Methods to rapidly and easily distinguish pathogenic isolates of *V. parahaemolyticus* from environmental reservoirs of mixed vibrio populations are necessary to (i) address seafood safety concerns; (ii) monitor the spread of virulent strains; and (iii) to study and understand the evolution of *V. parahaemolyticus*. Knowledge of pathogenicity traits and specifically the spread of virulent forms, may further enable the development of models that may be applicable to other pathogenic bacteria from the marine ecosystem. Following on from the methods comparison in the previous chapter, this discussion attempts to address the use and applications of the adopted strain differentiation methods of *V. parahaemolyticus* in a wider context in light of the findings presented in this thesis and recent literature.
11.2 Scope and feasibility of the present study

Although traditional biochemical tests and growth experiments proved to be labour intensive, they may still be useful because of some recent evidence to suggest biochemical heterogeneity amongst clinical and environmental isolates (Chapter 4; Martinez-Urtaza et al., 2006). This study aimed to apply existing molecular typing methods and to develop new ones to assess whether pathogenic *V. parahaemolyticus* could be differentiated from non-pathogenic isolates. It was envisaged that by targeting different loci with several typing systems, the study would (i) establish a measure of the diversity present in *V. parahaemolyticus* genomes; (ii) allow the identification of *V. parahaemolyticus* based on source; i.e., environmental or clinical, animal host or geographical location; and (iii) determine whether a proportion of environmental isolates may possess characteristic attributes similar to established virulent strains as a means for classifying potentially pathogenic isolates. However, the scope of the present work did not include establishing the pathogenicity status of any of the environmentally acquired isolates.

This study was conducted with a limited number of isolates but acquired from several sources (section 3.2.1) and therefore forms essentially a preliminary study of general molecular typing of *V. parahaemolyticus*. The methods described in this thesis should be validated on a larger population of isolates to assess the *in-vitro* stability of the ITS1-PCR and tDNA-ILP methods. The isolates used were acquired and typed over a period of three years (2003 to 2006). Based on a single laboratory user, and two PCR machines (section 3.6), the methods described seem stable, reproducible and applicable to other members of the *Vibrio* genus. The ease of use and reduced time required in comparison to techniques such as PFGE facilitated the generation of multiple fingerprint types with different primer pairs. Perhaps the most significant advantages of both ITS1-PCR and tDNA-ILP methods are their practicality, as the generation of a small number of amplification bands enables easier visual assessment without the need for complicated software analysis. If these
methods are to be further explored, a larger proportion of clinical isolates with more complete histories and from more diverse geographical sources would be recommended.

Based on the percentage of \textit{tdh} and \textit{trh} genotypes present in the isolates tested, it seems unlikely that the environmental isolates used in the study represent a true reflection of \textit{tdh} or \textit{trh} genes present amongst environmental reservoirs of \textit{V. parahaemolyticus} (section 10.2.2) and therefore, a bias towards potentially pathogenic strains. Such sample bias in favour of \textit{tdh}$^+$ isolates may initially have suggested greater relationships amongst those environmental and established clinical isolates. Although \textit{tdh}$^+$ isolates were found in several clusters, much the same as TTSS2 positive isolates (Table 9.5; Fig. 9.10) there appeared little evidence of environmental and clinical \textit{tdh}$^+$ isolates being grouped together. This would suggest that such haemolysin genes have been incorporated into the genome more recently. However, despite these differences there was some evidence to suggest that isolates obtained from the same shellfish species could be clustered using the methods described. For example, the majority of isolates sourced from oysters were found in separate groups to those isolates from other shellfish species. Isolates VP491, VP939 (obtained from scallop and crab respectively) were clearly differentiated from each other and the majority of those obtained from oysters.

11.3 Genome plasticity of \textit{V. parahaemolyticus}

The isolates were found to be from diverse O and K antigenic serotypes and this heterogeneity amongst \textit{V. parahaemolyticus} was clearly confirmed by PFGE analysis with \textit{NotI}. Amongst this heterogeneity, and as expected, environmental isolates accounted for the majority of the serotypes (70%). In both clinical and environmental cases, isolates of the same serotype showed distinct PFGE genotypes; conversely, isolates of similar PFGE patterns were of different serotypes. This phenomenon has been demonstrated widely in \textit{V. parahaemolyticus}, as up to 21 different serovariants have been found to be clonally related.
to the parent O3:K6 clone (Martinez-Urtaza et al., 2004; Nair et al., 2007). It has been suggested that the acquisition of additional serotypes may be a selected response to host immunological pressure (Chowdhury et al., 2004b). Such changes in the O- and K-antigens thereby clearly illustrate the fluidity of *V. parahaemolyticus*.

Based on the variations in length polymorphisms identified in both 16S-23S (ITS1) and between tRNA genes, it seems highly likely that some of these regions are undergoing large degrees of change in *V. parahaemolyticus* populations. Based on banding profiles alone, it appears that some of the tDNA regions varied more than others amongst environmental isolates. The largest amplicon with primers T3A/T5A (tD1) showed the greatest variation, whereas this amplicon appeared uniform in most of the clinical isolates (Fig. 7.2). By using another pair of discriminatory primers T3B/T5A (tD2) it was possible to confirm and establish the differences in relationships. Thus, the use of a secondary or even tertiary fingerprint pattern (i.e. tD3) would be essential to establish true diversity and hence more accurate relationships. The presence or absence of bands in the tDNA-ILPs suggests there may be rearrangements in the genome that need to be examined to assess for possible insertions of mobile genetic elements.

Of the typing systems employed, PFGE revealed the most variation in isolates and very recently, standardised methods that show increased reliability for typing, have been put forward (Wong et al., 2007; Kam et al., 2008). Variations in PFGE and RAPD profiles in bacteria are due to discrete insertions or deletions that contribute to restriction or primer site changes between strains (Kudva et al., 2002). In *E. coli*, it is thought that bacteriophage variation is a major factor in generating genomic diversity that underlies genomic variability detected by PFGE (Ohnishi et al., 2002). With the identification of an increasing number of non-host specific vibriophages (Matsuzaki et al., 2000; Millar et al.,
2003; Comeau et al., 2006) in the marine environment, it is likely that *V. parahaemolyticus* will continue to evolve at a relatively fast rate.

11.4 The differentiation of clinically significant *V. parahaemolyticus*

This work was based on the founding molecular typing principle that epidemiologically related isolates are derivatives of a single parent isolate (Streulens, 1998). Therefore, pathogenic isolates share characteristics that may differ from those of non-pathogenic isolates within the same species. The typing experiments carried out did not give a single distinct cluster of pathogenic *V. parahaemolyticus*. Clinical isolates were identified in several different clusters and in some cases clinical isolates formed single members (219, 224, 228 and E154482) of their own groups. PFGE analysis identified four members of the clonal pandemic O3:K6 group, which belonged to a larger cluster (>76% similarity) of 10 entirely clinical isolates. Another cluster (>76% similarity) contained five isolates, four of which showed β-haemolysis (an environmental and clinical isolate appearing closely related) and clustered with an environmental isolate, VP350 (Fig. 5.11). Similar findings were also observed in RAPD analysis. Whilst these methods clearly generate more diverse patterns than ITS1-PCR or tDNA-PCR, there appears evidence that more discriminate methods such as PFGE or ribotyping appear to correspond with strain source even less than lesser discriminate methods (Tamplin et al., 1996; Hoi et al., 1997; Chatzidaki-Livanis et al., 2006; Gonzalez-Escalona et al., 2006a). The inability to identify isolates of single pathogenic lineages, even by less discriminate methods such as ITS1-PCR and tDNA-ILP, may suggest that acquisition of pathogenicity by *V. parahaemolyticus* is a more recent event, occurring by lateral transfer. These findings would support current opinions on the acquisition of the TTSS2 by *V. parahaemolyticus* (Park et al., 2004b; Ono et al., 2006; Izutsu et al., 2008) and the hypothesis of a rapidly evolving pathogen.
The rise and dominance of clonally related O3:K6 *V. parahaemolyticus* (Chiou et al., 2000; Chowdhury et al., 2004b; Ansaruzzaman et al., 2005; Rizvi et al., 2006; Serichantalergs, et al., 2007) amongst infections reported worldwide has prompted the development of sequenced based methods (Gonzalez-Escalona et al., 2008). Presently, it is not clear why this particular clone and its derivatives have gained prevalence, although *in vitro* assessments have clearly shown enhanced virulence traits in comparative studies between O3:K6 related and non-O3:K6 related strains and is reviewed by Nair et al. (2007). Until the molecular basis of pathogenicity of *V. parahaemolyticus* is elucidated, it seems unlikely that there will be answers to such questions as, ‘what makes the O3:K6 related strains more dominant amongst clinical strains?’ This ‘pandemic’ isolate is thought to have been derived from the acquisition of the gene *tdh* by a non-pathogenic isolate (Okura et al., 2003), and this fact, together with the regional dominance of several other serovars both in Asia and the USA, suggests that a particular clone could gain dominance at any time.

Other than the increased hospitalisations due to the O3:K6 clonally related serovariants (Nair et al., 2007), to date there appear no reports on the severity of the illnesses associated with *V. parahaemolyticus* infections. This could be attributed to the lack of reporting due to the self-limiting nature of the infection and inadequate surveillance due to the present inability to differentiate clonally related *V. parahaemolyticus*. In clonal populations of *E. coli* O157:H7 it was found that a subpopulation belonging to clade 8 was responsible for contributing to more severe disease (Manning et al., 2008). This was identified based upon the analysis of 96 SNP loci and the identification of 39 SNP polymorphisms differing at 20% of the SNP loci. This present work has identified at least 34 polymorphic sites on the three structural genes of the TTSS1 region and hence indicates the potential of SNP typing of clonal populations to monitor the severity of disease and better surveillance of *V. parahaemolyticus*. Despite the large increase in infections resulting from clonally derived
O3:K6 strains, a recent review suggested that these clones do not seem to have the propensity for elevating hospital admissions to the same degree as pandemic isolates that were initially characterised in 1996. This led Nair et al., (2007) to speculate that there may be some degree of decay in the epidemic process. Thus, what was essentially considered a dominant serovar amongst infections seems to be on the decrease in hospital acquired infections in Calcutta, India. This would further support the concept that any one of the clones could gain dominance at any time, and highlights the need for increased vigilance.

11.5 Conclusions

Clearly, the scope of the work presented in this thesis is limited both in isolate numbers, and analytical methods employed. This study focussed on three techniques that up until now, had not been assessed in *V. parahaemolyticus* before: a two-primer RAPD method, ITS1-PCR and tDNA-ILP typing. The work formed the first of its kind in the UK in collaboration with the Community Reference Laboratory for monitoring bacteriological and viral contamination of bivalve molluscs (CEFAS). It is hoped that at least some of the findings would be explored further; possibly a blind inter-laboratory test which examines the usefulness of the RAPD markers to distinguish O3:K6 related isolates and the reproducibility of the typing methods in the hands of multiple users. Additional suggestions for further work have been described in previous chapters.

*V. parahaemolyticus* is genotypically a diverse organism. It appears pathogenic isolates possess diverse genotypes that stem from different lineages with evidence of recent lateral transfer of virulence determinants. Although most efforts over the last decade have centred on the epidemiology of the O3:K6 clonally related isolates, clinical disease can be caused by a wide variety of types. The ability for *V. parahaemolyticus* to survive and proliferate in diverse environmental conditions and exist as various cell types is clearly a result of its
genome fluidity. With its remarkably fast growth properties, not only is this a model organism to study bacterial growth, but also to study the evolution of marine bacteria. The development of simple and more robust typing systems are essential for the routine monitoring of this emerging pathogen. The present study has shed some light on the genome plasticity of *V. parahaemolyticus* yet many more questions have arisen and remain unanswered.
Appendix I

MEDIA, REAGENTS, SOLUTIONS AND SUPPLIERS
I.1 Bacteriological media

Tryptone soya broth (TSB)
TSB powder was purchased as pre-made media from Oxoid. 30g of media was dissolved in 1L of ddH2O, the pH adjusted to 7.5 and then autoclaved. The formula of the resulting media is as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone (casein digest USP)</td>
<td>17.0g</td>
</tr>
<tr>
<td>Soya peptone</td>
<td>3.0g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0g</td>
</tr>
<tr>
<td>Dibasic potassium phosphate</td>
<td>2.5g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.5g</td>
</tr>
</tbody>
</table>

Tryptone soy agar (TSA)
TSA powder was purchased as pre-made media from Oxoid. 37g of media was dissolved in 1L of ddH2O, 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.

Luria-Bertani medium (LB)
Per litre of ddH2O:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>10g</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
</tr>
<tr>
<td>Sterilised by autoclaving</td>
<td></td>
</tr>
</tbody>
</table>

LB agar was prepared as stated above but with the addition of 1.5% (w/v) agar

Marine salts broth (MSB)
Marine Broth 2216 powder was purchased as pre-made media from Becton Dickinson, distributors for Difco Labs. 37.4g of media was dissolved in 1L of ddH2O, boiled for 1 min to completely dissolve the powder and autoclaved.

Approximate formula per litre (pH 7.6 ± 0.2 @ 25°C)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.00 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1.00 g</td>
</tr>
<tr>
<td>Ferric Citrate</td>
<td>0.10 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>19.45 g</td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>5.90 g</td>
</tr>
<tr>
<td>Magnesium Sulphate</td>
<td>3.24 g</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>1.80 g</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>0.55 g</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>0.16 g</td>
</tr>
<tr>
<td>Potassium Bromide</td>
<td>0.08 g</td>
</tr>
<tr>
<td>Strontium Chloride</td>
<td>34.0 mg</td>
</tr>
<tr>
<td>Boric Acid</td>
<td>22.0 mg</td>
</tr>
<tr>
<td>Sodium Silicate</td>
<td>4.0 mg</td>
</tr>
<tr>
<td>Sodium Fluoride</td>
<td>2.4 mg</td>
</tr>
<tr>
<td>Ammonium Nitrate</td>
<td>1.6 mg</td>
</tr>
<tr>
<td>Disodium Phosphate</td>
<td>8.0 mg</td>
</tr>
</tbody>
</table>
Marine salts agar (MSA)
Marine Broth 2216 power (37.4g) of media was dissolved in 1L of ddH₂O with the addition of 1.5% w/v agar.

TCBS agar
TCBS Cholera Medium (CM0333) powder was purchased as pre-made media from Oxoid. 88g of media was dissolved in 1L of ddH₂O. The formula of the resulting media is as follows:

Yeast extract 5.00g
Bacteriological peptone 10.00g
Sodium thiosulphate 10.00g
Sodium citrate 10.00g
Ox Bile 8.00g
Sucrose 20.00g
Sodium chloride 10.00g
Ferric citrate 1.00g
Bromothymol blue 0.04g
Thymol blue 0.04g
Agar 14.00g

pH 8.6 ± 0.2 @ 25°C

Wagatsuma agar (WA) – M178

Yeast extract 3 g
Peptone 10 g
NaCl 70 g
K₂HPO₄ 5 g
Mannitol 10 g
Crystal violet 0.001 g
Agar 15 g
Distilled water 1 litre
Human or rabbit red blood cells, fresh (24 h), with anticoagulant 50 ml

Fresh rabbit blood was washed with same volume of physiological saline three times (Centrifuge cells at about 4000 x g at 4°C for 15 min each time).

All above ingredients, except blood, was suspended in distilled water and boiled to dissolve agar. The pH was adjusted to 8.0 and steam sterilised (30 min). A volume of 50 ml of washed red blood cells was added to the cooled (45°C) medium.
I.2 Genomic DNA isolation and purification

Bacterial genomic DNA was extracted using the ‘Puregene D-6000 DNA Isolation Kit’ manufactured by Gentra Systems Inc, Minneapolis, MN55441, USA. Details of the contents of each solution are as follows. Concentration of individual solution components is unknown.

**Cell Suspension Solution**
- Tris [hydroxymethyl] aminomethane
- EDTA
- Sorbitol

**Cell Lysis Solution**
- Tris [hydroxymethyl] aminomethane
- EDTA
- SDS

**Protein Precipitation Solution**
- Ammonium acetate

**DNA Hydration Solution**
- Tris
- EDTA

Lytic enzyme solution and RNase A stock solution were stored at 4°C. All other Puregene reagents were stored at room temperature (21°C).

I.3 General molecular biology reagents

**TBE buffer**
- TBE was made up at 5x strength according to the following formula and was then diluted to 1x:
  - TRIS-base: 54.0g
  - Boric acid: 27.5g
  - EDTA 0.5M [pH 8.0]: 20ml

**TE buffer**
- TE buffer was prepared either by diluting x 100 TE supplied by Sigma in sterile ‘Analar’ water or made up as a 1 x solution according to the following:
  - Tris-HCL: 10mM
  - EDTA: 1mM
  - pH 8
  - Autoclave
Gel loading solutions
(1) General gel loading (x 6) solution (Sigma):
Bromophenol blue 0.25% (w/v)
Xylene cyanole FF 0.25% (w/v)
Sucrose 40% (w/v)

(2) Tracking buffer (x 6) for RAPD (Amersham Biosciences)
30% Glycerol.
0.125% bromophenol blue
20 mM Tris-HCl (pH 8.0)

Roche Taq polymerase 10 x reaction buffer
Tris-HCl 100mM
MgCl₂ 15mM
KCl 500mM
pH 8.3

1.4 Pulsed field gel electrophoresis buffers
‘CHEF Bacterial Genomic DNA Plug Kit’ (BioRad) contained:
Cell Suspension Buffer (CSB)
600 U/ml Proteinase K,
Proteinase K Reaction Buffer
2% CleanCut Agarose
10x Wash Buffer
25 mg/ml Lysozyme,
Lysozyme Buffer

Contents and concentration of CSB and Wash Buffer are unknown.

Tris acetate EDTA
50 x stock:
Tris base 242g
Glacial acetic acid 57.1 ml
0.5M EDTA 100ml
pH 8

This solution was diluted immediately to a 1 x working concentration.

Tris-borate EDTA (TBE)
100 x stock:
Tris base 108g
Boric acid 55g
0.5M EDTA 40ml
pH 8

This solution was diluted immediately to a 0.5 x working concentration.
1.5 DNA purification kit solutions

DNA was purified using the ‘High Pure PCR Product Purification Kit’ supplied by Roche. Details of the content of each solution are as follows:

**Binding Buffer**
3 M guanidine-thiocyanate,
10 mM Tris- HCl,
5% ethanol (v/v),
pH 6.6 (25° C)

**Washing Buffer**
(final concentrations after addition of 40ml ethanol)
20 mM NaCl,
2 mM Tris-HCl, pH 7.5 (25°C)

**Elution Buffer**
10 mM Tris-HCl,
pH 8.5 (25°C)

1.6 Description of blotting solutions and buffers (Chapter 9)

**Depurination solution**
Per litre of ddH₂O:
HCl 0.2M

**Denaturing solution**
Per litre of ddH₂O:
NaCl 0.5M
Tris-HCl 1.5M
pH 8

**Neutralisation solution**
Per litre of ddH₂O:
NaOH 1.5M
NaCl 0.5M

**20 x SSC**
Per litre of ddH₂O:
Sodium chloride 175.32g
Sodium citrate 88.23g
pH 7

The above solution was diluted to 10 x, 5 x, 2 x, or 0.1 x strength as required with sterile ‘Analar’ water.
1.7 DNA Labelling and Detection (Chapter 9)

DNA was labelled using the ‘DIG DNA Labelling and Detection Kit’ supplied by Roche. Details of the content of each solution are as follows:

**Unlabelled control DNA 1**
20 µl pBR328, 100 µg/ml
pBR328 is digested separately with BamHI, BglI and HinfI

**Unlabelled control DNA 2**
20 µl pBR328, 200 µg/ml
Linearised with BamHI

**Labeled Control DNA**
50 µl pBR328 DNA (5 µg/ml)
Linearised with BamHI

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

**Hexanucleotide Mix (10 × concentrated hexanucleotide reaction mix)**
Random hexanucleotides 62.5 A₂₆₀ units/ml (1.56mg/ml)
Tris-Cl 500mM
MgCl₂ 100mM
Dithioerythritol (DTE) 1mM
BSA 2mg/ml
pH 7.2

**dNTP labelling mixture**
10 × concentrated dNTP labeling mix containing:
1 mM dATP
1 mM dGTP
1 mM dCTP
0.65 mM dTTP
0.35 mM DIG-11-dUTP
pH 7.5 (20°C)

**Klenow enzyme, labelling grade**
Klenow enzyme (2 U/µl)

**Anti-Digoxigenin-AP Conjugate**
Polyclonal sheep anti-digoxigenin
Fab-fragments, conjugated to alkaline phosphatase
750 U/ml

**NBT/BCIP**
Concentrated stock solution (exact concentration unknown)
**Buffer 1** - Washing Buffer
0.1 M Maleic acid
0.15 M NaCl
pH 7.5 (20° C)
0.3% (v/v) Tween 20

**Buffer 2** - Blocking reagent
(content unknown)
10x stock and 1x working solutions were prepared with maleic acid buffer

**Buffer 3** - Alkaline phosphatase detection buffer
M Tris-HCl, 0.1 M NaCl, pH 9.5 (20° C)

**Maleic acid buffer** – for dilution of blocking reagent
0.1 M Maleic acid
0.15 M NaCl
Adjusted with NaOH (solid) to pH 7.5 (20° C)

**TE-buffer**
10 mM Tris-HCl
1 mM EDTA
pH 8.0

**Stripping and reprobing of DNA blots**
Dimethylformamide (DMF)
0.2 N NaOH, 0.1% SDS (w/v), 2 x SSC
1.8 Molecular weight markers

**TrackIt™ 50 bp DNA Ladder** Gibco (Invitrogen)  
0.5 μg/lane

The 50 bp ladders range from 50-800 bp in 50 bp increments, plus a 2652 bp fragment. The 350 bp band is ~ 2 times brighter than the others.

Tracking dyes, Xylene Cyanol FF (XCFF) and Orange G, allows tracking of DNA migration during electrophoresis and indicate when maximum resolution is achieved.

**100 bp Ladders 100 – 2072bp** Gibco (Invitrogen)  
0.5 μg/lane

The 100 bp ladders range from 100-1500 bp in 100 bp increments, plus a 2072 bp fragment. The 600 bp band is 2-3 times brighter than the others.

During 2% agarose gel electrophoresis with tris-acetate (pH 7.6) as the running buffer, bromophenol blue migrates near the 100-bp fragment.
1kb Ladder 75 – 12,216bp Gibco (Invitrogen)
0.5 µg/lane

The 1kb ladders range from 75bp to 12,216 bp in various increments.

A: Lambda Ladder 48.5 – 873 kb (BioRad)
B: *Saccharomyces cerevisiae* Ladders 0.2 – 2.2 MB (BioRad)

A: Lambda ladders (concatemers of \(\lambda\)cl857sam7) range from 48.5kb – 873 kb in 48.5kb increments purchased pre-prepared.

B: *Saccharomyces cerevisiae* size standards range from 0.225 – 2.20 MB.
1.9 Suppliers

Becton Dickinson
The Danby Building
Edmund Halley Road
Oxford Science Park
Oxford
OX4 4DQ
http://www.bd.com

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

Gentra Systems (now Qiagen Ltd.)
13355 10th Avenue North
Suite 120
Minneapolis
MN55441
USA

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

MWG Biotech
Eurofins MWG Operon
318 Worple 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
Tel: 01293-422-911
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
II.1 Bacterial DNA quantification

The concentration of extracted genomic DNA was established by agarose gel electrophoresis (Section 3.8). The DNA concentration was estimated for each isolate by comparison with a range of the following known amounts of λ DNA standards indicated in Figure II.1 using UV photo software (UViTec, UK) (Fig. II.1). For confirmation and quantification of DNA of additional isolates, genomic DNA was quantified with a UV spectrophotometer (NanoDrop 100).

![Image of agarose gel electrophoresis](image)

**Figure II.1**: Quantification of DNA extracted from *V. parahaemolyticus* and other bacterial cultures.

**Panel A**: lanes 1-10 correspond to the following, 1; 280ng of λ standard DNA, 2; 140ng of λ DNA standard, 3; 70ng of λ standard DNA, 4; 35ng of λ standard DNA, 5; DNA from isolate 1103/9, 6; DNA from isolate VC2, 7; DNA from isolate Vib3, 8; DNA from isolate VP6, 9; DNA from isolate Vib6, 10; DNA from isolate VC5.

**Panel B**: lanes 1-10 correspond to the following, 1; 280ng of λ standard DNA, 2; 140ng of λ DNA standard, 3; 70ng of λ standard DNA, 4; 35ng of λ standard DNA, 5; DNA from isolate 6134, 6; DNA from isolate VP10884, 7; DNA from isolate Vib5, 8; DNA from isolate VP8, 9; DNA from isolate VC1, 10; DNA from isolate 1022/10.
Panel C: lanes 1-10 correspond to the following, 1; 280ng of $\lambda$ standard DNA, 2; 140ng of $\lambda$ DNA standard, 3; 70ng of $\lambda$ standard DNA, 4; 35ng of $\lambda$ standard DNA, 5; DNA from isolate Vib1, 6; DNA from isolate 1023/1, 7; DNA from isolate VP361, 8; DNA from isolate Vib2, 9; DNA from isolate 11327, 10; DNA from isolate VP491, 11; DNA from isolate VP9.

Panel D: lanes 1-10 correspond to the following, 1; DNA from isolate VM499, 2; DNA from isolate VP331, 3; DNA from isolate VP349, 4; DNA from isolate Vib7, 5; DNA from isolate E154482, 6; DNA from isolate VA430, 7; DNA from isolate 8042, 8; DNA from isolate Vib10, 9; DNA from isolate VP346, 10; DNA from isolate E168143, 11; 140ng of $\lambda$ standard DNA.

Panel E: lanes 1-10 correspond to the following, 1; 280ng of $\lambda$ standard DNA, 2; 140ng of $\lambda$ DNA standard, 3; 70ng of $\lambda$ standard DNA, 4; DNA from isolate PM5, 5; DNA from isolate KA5, 6; DNA from isolate LD365, 7; DNA from isolate EC5, 8; DNA from isolate Vib4, 9; DNA from isolate ST5, 10; DNA from isolate EA5, 11; DNA from isolate E155855.
Figure II.1: Cont.

Panel F: lanes 1-10 correspond to the following, 1; 280ng of λ standard DNA, 2; 140ng of λ DNA standard, 3; 70ng of λ standard DNA, 4; DNA from isolate 2053, 5; DNA from isolate 2341, 6; DNA from isolate KIDBVV, 7; DNA from isolate Vib3, 8; DNA from isolate 5421, 9; DNA from isolate VP416, 10; DNA from isolate VP378, 11; DNA from isolate Vib5.

Panel G: lanes 1-10 correspond to the following, 1; 280ng of λ standard DNA, 2; 140ng of λ DNA standard, 3; 70ng of λ standard DNA, 4; 35ng of λ standard DNA, 5; 17 ng of λ standard DNA, 6; DNA from isolate 219, 7; DNA from isolate 220, 8; DNA from isolate 221, 9; DNA from isolate 222, 10; DNA from isolate 223.

Panel H: lanes 1-10 correspond to the following, 1; 280ng of λ standard DNA, 2; 140ng of λ DNA standard, 3; 70ng of λ standard DNA, 4; 35ng of λ standard DNA, 5; 17 ng of λ standard DNA, 6; DNA from isolate 224, 7; DNA from isolate 225, 8; DNA from isolate 226, 9; DNA from isolate 227, 10; DNA from isolate 228.

Note: Plates C – F are scanned digitised images
Table II.1 Genomic DNA concentrations determined from agarose gels. Isolates not in Fig. II.1 were quantified with a UV spectrophotometer. DNA from other species were purchased lyophilised and of known concentrations.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>DNA conc. (ng/μl)</th>
<th>Isolate</th>
<th>DNA (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1902</td>
<td>20</td>
<td>E154482</td>
<td>60</td>
</tr>
<tr>
<td>8042</td>
<td>50</td>
<td>E155855</td>
<td>50</td>
</tr>
<tr>
<td>11327</td>
<td>100</td>
<td>E168143</td>
<td>70</td>
</tr>
<tr>
<td>1021/5</td>
<td>50</td>
<td>VP331</td>
<td>70</td>
</tr>
<tr>
<td>1022/10</td>
<td>280</td>
<td>VP345</td>
<td>40</td>
</tr>
<tr>
<td>1023/1</td>
<td>30</td>
<td>VP346</td>
<td>70</td>
</tr>
<tr>
<td>1023/3</td>
<td>150</td>
<td>VP349</td>
<td>70</td>
</tr>
<tr>
<td>1024/5</td>
<td>250</td>
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<td>60</td>
</tr>
<tr>
<td>1103/9</td>
<td>30</td>
<td>VP361</td>
<td>100</td>
</tr>
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</tr>
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</tr>
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<td>VP491</td>
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</tr>
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<td>228</td>
<td>80</td>
</tr>
<tr>
<td>Cla</td>
<td>10</td>
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<td></td>
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### II.2 Phenotypic growth characteristics of *V. parahaemolyticus*

Table II.2: Growth characteristics of *V. parahaemolyticus* on non-selective and selective solid media (Chapter 4)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Environmental/Clinical/Reference strain</th>
<th>Growth media¹</th>
<th>MSA</th>
<th>TSA</th>
<th>WA</th>
<th>TCBS</th>
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<td>NCIMB Reference Strain</td>
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<td>+++</td>
<td>+</td>
<td>+++</td>
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<td>VP10884</td>
<td>NCTC Reference Strain</td>
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<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>VP10885</td>
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<td></td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
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<td>219</td>
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<td>+++</td>
<td>+++</td>
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<td>+++</td>
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<td>221</td>
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<td></td>
<td>+++</td>
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¹ Environmental and Clinical isolates were tested on non-selective and selective solid media.
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¹ Growth on media:
MSA: Marine salts agar (Marine Agar)
TSA: Tryptic soy agar (3% NaCl)
WA: Wagatsuma agar
TCBS: Thiosulphate citrate bile salts agar

Growth was monitored at 18 hours and scored as, +++ Very good, ++ Good, + Poor, - No growth

Visible exopolysaccharide production in Wagatsuma agar present only in isolates VPEC1 and VPSC1
Table 11.3: Comparison of growth rates of clinical and environmental isolates at 0.5 and 3% NaCl in TSB, pH 7.2. Doubling times were obtained by monitoring absorbance at 600nm and graphical plots of log absorbance (y) and time (x). Statistical significance testing (t-test) was carried out on the means and variance of data with NCSS statistical software analysis. Box plots (section 4.3.2) show variability of data.

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Appendix III

SEQUENCE DATA
Ill.1: SEQUENCING DATA RELEVANT TO CHAPTER 9

_Vibrio parahaemolyticus_ strain RIMD2210633 sequence information retrieved from: [http://genome.naist.jp/bacteria/vpara/index.html](http://genome.naist.jp/bacteria/vpara/index.html) and also available with GenBank accession numbers BA000031 and BA000032. Primer binding sites (underlined) for TTSS1 and TTSS2 genes are shown below (Figs. Ill.1 – Ill.6)

>VP1662
GTGAATCTCATGAATTGATCATATTTACTAATACGATGTTTATTGATGTTTTTGCCTTTGCCAACTGCACTAGTGGATGCTGATCGGCGCAAACATGAGTATTGCCGTCGTGTTGCTGATGTTGGCGATTTACATCACCACGCCACTGGAGTTTTCGGCGTTTCCGGCAGTATTGTTGATAACGACGCTATTTCGTTTGTCCCTGTCTATCACCACGACACGTTTGATCCTGCTTCAAGGCGATGCGGGTCAAATCGTTTACACCTTTGGTAACTTTGTTGTTGGCGGCAACCTTGTCGTTGGTATCTGGGGTCTTCCTGATCATAACTATTGTACAGTTTATGGTGATCACCAAAGGTTCGGAGCGTGTTGCTGAAGTTAGTGCTCGATTCTCTCTTGATGCCATGCCGGGCAAACAGATGAGTATCGACGGTGACATGCGCGCTGGAGTTATCGATGTTCACGAGGCGCGTCATCGCCGTTCTCTGATCGAGAAAGAGAGTCAAATGTACGGCTCGATGGACGGCGCAATGAAGTTCGTAAAAGGC
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FigureIll.1: Complete nucleotide sequence of _vcrDI_. Low calcium response TTSS1 structural protein. PCR product size: 458 bp.
Figure III.2: Complete nucleotide sequence of *vp1680*. ‘Cytotoxic effector’ protein. PCR product size: 183 bp.
Figure III.3: Complete nucleotide sequence of vscC1. TTSS1 structural protein. PCR product size: 415 bp.
Figure 31.4: Complete nucleotide sequence of vpa1321. Cytotoxic necrotizing factor.
PCR product size: 485 bp.

Figure 31.5: Complete nucleotide sequence of vscC2. Putative TTSS2 structural protein.
PCR product size: 272 bp.
Figure III.6: Complete nucleotide sequence of vcrD2. Putative TTSS2 structural protein. PCR product size: 388 bp.
Figure III.7: Nucleotide sequences and multiple alignments of the \( \text{vpl680} \) locus of TTSS1 in isolates of \( V. \text{parahaemolyticus} \). RIMD2210633 is a sequenced clinical strain. Sequences AQ3810B and 220B are from clinical strains and VP416B from an environmental isolate. 1902B is NCIMB reference strain from which probe was developed. Consensus regions are shown in red; blue and black show substitutions or deletions. Isolate VP416 shows Regions marked in dotted line represent quality control clipped regions. SNPs in TTSS1 are also present in \( \text{verD1} \) and \( \text{vscC1} \).
Figure III.8: Nucleotide sequences and multiple alignments of the vseCJ locus of TTSS1 in isolates of V. parahaemolyticus. RIMD2210633 is a sequenced clinical strain. Sequences AQ3810C and 220C are from clinical strains and VP416C from an environmental isolate. 1902C is NCIMB reference strain from which probe was developed. Consensus regions are shown in red; blue and black show substitutions or deletions. Regions marked in dotted line represent quality control clipped regions. SNPs in TTSS1 are also present in verD71 and vp1680.
Figure III.9: Nucleotide sequences and multiple alignments of the \textit{vscC2} locus of TTSS2 in isolates of \textit{V. parahaemolyticus}. RIMD2210633 is a sequenced clinical strain. Nucleotide sequence 220E is from a clinical strain and VP416B from an environmental isolate. Consensus regions are shown in red; blue and black show substitutions or deletions. Regions marked in dotted line represent quality control clipped regions. Regions within clipped areas were homologous in all three TTSS2 loci.
Figure III.10: Nucleotide sequences and multiple alignments of the vcrD2 locus of TTSS2 in isolates of *V. parahaemolyticus*. RIDM2210633 is a sequenced clinical strain. Nucleotide sequence 220E is from a clinical strain and VP416B from an environmental isolate. Consensus regions are shown in red; blue and black show substitutions or deletions. Regions marked in dotted line represent quality control clipped regions. Regions within clipped areas were homologous in all three TTSS2 loci.


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