Assessment of Methodologies for the Enumeration of Pathogenic *Vibrio* species in Australian Prawns

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Prawn Market Access Defenders

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Contents

Executive Summary

The *Vibrio* species most commonly detected in trade border control measures and human health outbreaks are *V. cholerae, V. parahaemolyticus* and *V. vulnificus*. These species were responsible for 50% of border detentions for crustacea into the EU market between 2008 and 2013. Increased international attention on these species has led to the Codex Committee on Food Hygiene (CCFH) advancing international standards for *Vibrio* spp. in seafood. The importance of these pathogens for prawns in international trade is reflected by Australia setting import requirements for *V. cholerae*. Further, some major retailers are setting microbiological requirements for specific *Vibrio* species and these requirements are becoming more stringent.

Changing international and domestic policies leave the Australian Prawn Industry exposed to some risk. The increased international attention on the presence of pathogenic vibrios in seafood is a significant risk as it will likely result in a larger proportion of product detentions and rejections due to *Vibrio* species in prawns. Considering the lack of any epidemiology or trade detentions associated with the consumption of Australian prawns, there is some potential that appropriate methodologies may help differentiate Australian prawns in the international market place as being of a low risk for pathogenic *Vibrio* species. This work aimed to review all the available methods to identify the most appropriate method for use in determining the presence of pathogenic *Vibrio* species in Australian prawns. Based on this information, the review suggests ways to improve and harmonise approaches to *Vibrio* testing of seafood.

Based on the review of available methodologies, four methods for detecting these pathogens were chosen for further analysis: conventional PCR, Real-Time PCR, Loop-Mediated Isothermal Amplification (LAMP) and DNA hybridisation. The main considerations when selecting a method are to assess if they are specific, robust, repeatable, cost effective and rapid. These aspects were considered by establishing and assessing these four methods in our laboratory. Real-Time PCR achieved the highest score on all considerations and also included an internal amplification control (IAC). The IAC provides the additional safety of ensuring there is no inhibition of amplification from the sample, a common problem with seafood samples. For these reasons, Real-Time PCR was selected as the most appropriate method and this method was used for the final stages of the project.

To assess the specificity of the Real-Time PCR methods selected, a library of known *Vibrio* strains was developed (containing 197 *Vibrio* strains). This included both Australian and International *Vibrio* strains with both target and non-target species of *Vibrio*. Each duplex Real-Time PCR method targeting *ctx* (*V. cholerae*), *tlh, tdh, trh* (*V. parahaemolyticus*), and *vvh* (*V. vulnificus*) was assessed against DNA extracts from 96 library strains, including both target and non-target species. Each method was demonstrated to be 100% specific for the target species. Such an outcome will provide a high level of confidence in future results from prawn samples where a wide range of *Vibrio* species may be present.

Following the specificity assessment, the robustness of the methods was assessed. Firstly, the duplex methods targeting species-specific genes were assessed against either mixed cultures (*V. parahaemolyticus* and *V. vulnificus*) or mixtures of extracted DNA (*V. cholerae*) to assess robustness. All methods correctly identified the target species. To demonstrate that the selected methods showed linearity with increasing concentrations of the target species when the most probable number technique was employed, green prawn meat samples were

contaminated in triplicate with known amounts of either *V. parahaemolyticus* or *V. vulnificus* (*V. cholerae* could not be assessed due to legal limitations on laboratory cultures in Australia). A regression analysis against the known concentrations showed linearity (R^2 values of 0.92 and 0.91 for *V. parahaemolyticus* and *V. vulnificus* respectively) to the results achieved by real-time PCR.

Taken together, these results indicate that the methods identified and assessed as part of this work, are specific, sensitive and robust and are appropriate for use in assessing for the presence or absence of these species in Australian prawns. However, a final point of method validation is required in the future in the form an inter-laboratory study. There are currently discussions to complete this with the USFDA.

The availability of this method to the Australian prawn industry will allow them to investigate the pathogenicity of any positives from import/retail. This is in line with national, international and Codex policies relating to pathogenic *Vibrio* species in seafood. Therefore, consideration should be given to entering into discussions with the relevant authorities and testing laboratories in Australia on the integration of the methods into existing monitoring programs and the utilisation of such methods to underpin a risk assessment for Australian prawns. Further consideration should also be given to providing this report to Australian analytical laboratories for the potential uptake of the methods described.

A small survey of raw Australian prawns was undertaken. All prawn samples tested had <0.3 MPN/g of *V. cholerae* and *V. vulnificus*. All but one sample were found to be below the level of detection (<0.3 MPN/g) for *V. parahaemolyticus*. The one positive sample, a Western King Prawn sample from South Australia, had 0.3 MPN/g. The sample did not contain the *V. parahaemolyticus* virulence marker *tdh* or *trh*. These data confirm the low prevalence of pathogenic *Vibrio* species in Australian prawns.

Introduction

The *Vibrio* species most commonly detected in trade border control measures and human health outbreaks are *V. cholerae, V. parahaemolyticus* and *V. vulnificus*. As many as 50% of border detentions for crustacea into the EU market between 2008 and 2013 were due to pathogenic *Vibrio* species (http://ec.europa.eu/food/food/rapidalert/index_en.htm). Furthermore, among prawns imported into the EU between 1980 and 2010 there were 112 alerts for *V. parahaemolyticus* and 50 for *V. cholerae* (Sumner, 2011).

Multiple countries now set standards for these species including China, Japan, Thailand and the United States of America. Australia does not currently set a domestic standard for any *Vibrio* species. However, it applies an import requirement for prawns and shrimp for *V. cholerae.* This requirement also applies to Australian product that has been exported for processing prior to re-importation into Australia. In addition, some major retailers in Australia set microbiological requirements for some *Vibrio* species and these requirements are becoming more stringent. The 2009 Woolworths Quality Assurance Standard (Version 6) specified that *V. cholerae* should not be detected in one gram of either cooked or uncooked crustacea. In Version 9 of this document, released in March 2013, the stringency of this standard has increased to *should not be detected in 25 grams*. These changes between versions highlight the increasing attention being applied to these pathogens.

Codex Alimentarius is an international body that sets food safety policies. Membership of Codex is open to all Member Nations and Associate Members of the Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO). Currently, Codex members cover 99% of the world population. Codex documents contribute to the safety, quality and fairness of the international trade in food. The Codex Committee on Food Hygiene (CCFH) is currently advancing international standards for *Vibrio* spp. in seafood. These guidelines were adopted in May 2011 and suggest that test methods should distinguish between virulent and non-virulent strains. Australia aligns their domestic standards with those set down by Codex to ensure consistency with World Trade Organisation (WTO) policies. Consequently, there is an expectation that Australia will eventually adopt the guidelines set down by the CCFH on pathogenic vibrios in seafood. Although, in the finalised version (CAC/GL 73-2010) there is no requirement for monitoring for these species in prawns. However, the introduction of this Guideline will likely maintain the international focus on these species.

Considering the increased attention both internationally and domestically, it is a concern that accredited laboratory methods for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* in Australia are based on standard microbiological techniques. These methods are time consuming and don't differentiate between 'disease-causing' and 'non-disease causing' strains and hence can over score the risk. The lack of a rapid capability that distinguishes virulent and non-virulent strains means that Australia is not able to effectively or efficiently dispute *Vibrio* related prawn trade issues. Although it should be noted that the regulatory framework for these pathogens with accepted molecular methodologies to detect *V. cholerae* focus mainly on the virulence-associated factor *ctx* rather than a species-specific gene.

The changing international and domestic dynamics leave the Australian prawn industry exposed to market access issues related to this species. The increased international attention is a significant risk as it will likely result in a larger proportion of product detentions and rejections for vibrios in prawns and possibly increased costs to industry in compliance

testing. However, considering the lack of any epidemiology or trade detentions associated with the consumption of Australian prawns, there is some potential that appropriate methodologies may help differentiate Australian prawns in the international market place as being of a low risk for pathogenic *Vibrio* species.

Aims

1. Conduct a literature review to establish all current methods that have been implemented for the identification and enumeration of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*.
2. Determine the most suitable of these methods for the detection of pathogenic vibrios in prawns.
3. Select an appropriate methodology and assess its specificity and robustness.
4. Undertake a short preliminary survey of Australian prawns.
5. A recognised capacity to assist with potential public health and trade issues related to *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in prawns.

1 Literature Review

The bacterial genus *Vibrio* contains a number of pathogens that are capable of causing disease in humans (Desmarchelier, 2003). *Vibrio* species are halophilic, facultative anaerobic, Gram-negative bacteria belonging to the *Gamma-proteobacteria* class of bacteria. They are straight or curved rod-shaped bacteria, about 1.4-2.6 μm long, motile by a single polar flagellum (Tantillo *et al*., 2004) and are usually oxidase positive (Murray *et al*., 1984). The genus currently contains more than 74 species (Noguerola & Blanch, 2008) and its taxonomy is continuously updated as new techniques allow the detection and identification of new species (Tantillo *et al*., 2004). *Vibrio* species are ubiquitous in marine environments (Arias *et al*., 1999; Hervio-Heath *et al*., 2002; Maxwell *et al*., 1991; Urakawa *et al*., 1999) and are found as planktonic cells and in association with sediments and other surfaces.

Various studies have demonstrated that there is significant seasonal variation in the numbers of *Vibrio* spp. within the water column. Numbers are generally highest when the water temperature is between 20°C and 30°C with *Vibrio* spp. of clinical significance generally not present when the temperature drops below 10°C (Tantillo, 2004). A link has also been demonstrated between salinity and the presence of virulent *Vibrio* spp. (Yam *et al*., 1999). However, most species are only found in water with salinities covering the range 5 – 36 ppm. Some observations indicate that they may survive outside of this range in the presence of high water nutrient content or high water temperature.

The Codex Committee for Fish and Fish Products has developed a set of guidelines to ensure safety of seafood products with respect to vibrios that can be expected to be adopted by seafood import/export countries. In light of this, an increased focus will be to assess the risk to consumers by different seafood commodities worldwide. However, there are a multitude of methods that can be applied to achieve this goal to assess risk and gather data on the presence and numbers of vibrios, and their pathogenicity, that need to be gathered and considered.

Current methods set by the international Organisation for Standardisation (ISO) for the detection and enumeration of *Vibrio* spp. (ISO 21872-1 and ISO 21872-2) are not considered appropriate to meet the criteria set by Codex Alimentarius as they are based on biochemical identification, which is time consuming, and do not determine likely virulence. Given the recent Codex recommendations and heightened international need to address risks from *Vibrio* spp. in seafood, it is essential that recommendations are made to assist industry in selecting and implementing appropriate methods of analysis.

This review aims to provide an overview of the current available methods and their suitability for meeting current and future international regulations for seafood safety, which will also be described. Based on this information, the review suggests ways to improve and harmonise approaches to *Vibrio* testing of seafood.

1.1 Methodologies for Identification of *Vibrio* spp.

There are a multitude of research articles in the literature describing novel methods for the specific detection and/or identification of virulent *Vibrio* spp. in a food sample. These articles describe methods including: species specific growth media, biochemical identification, serological identification, DNA hybridisation and/or genotypic identification (e.g. by polymerase chain reaction). There are also many methods described in the literature to

determine the potential virulence of these organisms present in a food sample. Here, we describe the different approaches taken for each of these methods and highlight the potential pros and cons of each approach.

1.1.1 Growth Media

Qualitative enrichment is commonly used as a first step in the enumeration and/or identification of virulent *Vibrio* spp.. The most common enrichment media used for this purpose is alkaline peptone water (peptone (1% w/v), NaCl (1% w/v), pH 8.5-9) (Wong *et al*., 1999). Whilst this media is non-selective, it provides a way of cultivating alkalophilic micro-organisms, such as *Vibrio* spp., that can then be used for further analysis.

There are also two solid growth media that are commonly used to specifically select for *Vibrio* spp.. The first is Thiosulphate-Citrate-Bile Salts-Sucrose (TCBS) agar (McCormack *et al*., 1974). This medium uses thiosulphate and bile salts to select for *Vibrio* spp. and citrate fermentation, and can further distinguish between sucrose positive and sucrose negative *Vibrio* species. *V. parahaemolyticus* and *V. vulnificus* appear as small lime green colonies, *V. cholerae* appears as large (2-4 mm diameter), circular, flattened, opaque yellow, and sticky colonies. However, it is not possible to make species identifications based solely on growth on TCBS due to similarity of colony morphologies with other *Vibrio* species. Therefore, further confirmatory tests are generally required to determine their identification. Importantly, the presence of bile salts in the growth medium may also be able to revive *V. parahaemolyticus* from the viable but non-culturable (VBNC) state (Pace *et al*., 1997), although it has also been reported that TCBS may inhibit the growth of some virulent strains (Raghunath *et al*., 2008).

CHROMagar-Vibrio is a chromagenic selective agar for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Hara-Kudo *et al*., 2001). Unlike TCBS agar, CHROMagar is able to distinguish *V. parahaemolyticus* by colour (mauve) compared to the other two species (green blue to turquoise blue). Most other species of bacteria will not grow on this media, but some produce a different colony morphology (e.g. *V. alginolyticus* will grow on this media but appear white/translucent). Further analysis is required to distinguish between *V. cholerae* and *V. vulnificus* isolated on this medium due to the similarity in colony morphology.

Typically, both of these media are used at 35-37°C as use of this temperature may assist in the selective growth of potentially virulent species (Hara-Kudo *et al*., 2001, McCormack *et al*., 1974).

1.1.1.1 V. cholerae

A range of media have been reported for the propagation of *V. cholerae (*Table 1).

Table 1: Media commonly used for the selective isolation of *V. cholerae*.

Medium	Abbreviation	Selective Agent	Reference
Monsur's Taurocholate Tellurite Peptone	TTP	pH (9.2), taurocholate, tellurite	Monsur, 1963
Starch-Gelatin-Polymyxin Broth*	SGP	Polymyxin B	Kitaura *et al*., 1983
CHROMagar-Vibrio*	N/A	Chromagenic Mix	Hara-Kudo *et al*., 2001
Monsur's Gelatin Taurocholate Agar	GTT	pH (8.5), taurocholate, tellurite	Monsur, 1961
Polymyxin Mannose Tellurite Agar	PMT	Polymyxin, tellurite, SDS	Shimada *et al*., 1990
SDS Polymyxin Sucrose Agar*	SDS; SPS	SDS, polymyxin B	Kitaura *et al*., 1983
Cellobiose Polymyxin Colistin Agar*	CPC	Polymyxin, colistin	Massad & Oliver, 1987
Modified CPC Agar*	mCPC	Polymyxin, colistin	Tamplin *et al*., 1991
Cellobiose Colistin Agar	CC	Colistin	Høi *et al.*, 1998

* Denotes media is also used for detection/enumeration of *V. vulnificus*

1.1.1.2 V. parahaemolyticus

Common selective media used for isolation of *V. parahaemolyticus* (Table 2) include glucose teepol salt broth (GTSB), salt polymyxin broth (SPB) or salt colistin broth (SCB). The selectivity of TCBS and Chromagar-Vibrio can be enhanced by a two stage enrichment process using salt trypticase soy broth (non-selective) and sub-culturing into salt polymyxin broth (Hara-Kudo *et al*., 2001). The authors found that the two-step enrichment provided more efficient recovery of *V. parahaemolyticus* and resulted in higher recovery from seafood than TCBS alone.

Table 2: Media commonly used for the selective isolation of *V. parahaemolyticus*

Medium	Abbreviation	Selective Agent	Reference
Water Blue-Alizarin Yellow Agar	WBAY	Teepol, NaCl, alizarin yellow	Sakazaki, 1963
Trypticase Soya Agar Triphenyl Tetrazolium	TSAT	Bile salts, NaCl	Kourany, 1983
CHROMagar-Vibrio	N/A	Chromagenic mix	Hara-Kudo *et al*., 2001
Glucose Salt Teepol Broth	GSTB	pH, methyl violet, teepol	Sakazaki *et al*., 1968
Water Blue-alizarin yellow Broth	WBAY	Teepol, water blue, alizarin yellow	Beuchat, 1977
Salt Polymyxin Broth	SPB	Colistin, NaCl	ISO, 1990
Salt Colistin Broth	SCB	Colistin, NaCl	Cowan, 1974
7% NaCl Tryptic Soy Broth	TSBS	7% NaCl	Beuchat, 1977

1.1.1.3 V. vulnificus

There are a number of growth media that are specifically designed for the selective growth of *V. vulnificus* (Table 3). These are predominantly based on the ability of this organism to

utilise cellobiose as a carbon source (VV Agar) and its resistance to polymyxin B and colistin. *V. cholerae* is also resistant to polymyxin B and colistin but cannot utilise cellobiose, and so appears purple with a blue halo on these media, compared to yellow colonies with yellow halos produced by *V. vulnificus* (Massad & Oliver, 1987). The addition of magnesium and potassium to these media has also been reported to increase recovery (Cerdá-Cuéllar *et al*, 2000).

Vibrio vulnificus agar is another selective agar based on this organism's ability to utilise salicin and tellurite (Brayton *et al*., 1983). *V. vulnificus* colonies appear light grey translucent with dark grey or black centres. While Brayton *et al*. (1983) report that this agar is selective, Massad and Oliver (1987) suggest that it may in fact allow for the growth of other *Vibrio* sp.

Table 3: Media commonly used for the selective isolation of *V. vulnificus*

Medium	Abbreviation	Selective Agent	Reference
Starch-Gelatin-Polymyxin Broth*	SGP	Polymyxin B	Kitaura *et al*., 1983
SDS Polymyxin Sucrose Agar*	SDS; SPS	SDS, polymyxin B	Kitaura *et al*., 1983
Cellobiose Polymyxin Colistin Agar*	CPC	Polymyxin, colistin	Massad & Oliver, 1987
CHROMagar-Vibrio*	N/A	Chromagenic Mix	Hara-Kudo *et al*., 2001
Modified CPC Agar*	mCPC	Polymyxin, colistin	Tamplin *et al*., 1991
Peptone NaCl Cellobiose Colistin Broth	PNCC	pH, colistin	Hsu *et al*., 1998
VV Agar	VV	Potassium tellurite, crystal violet, oxgall, pH (8.6)	Brayton *et al*., 1983
VVM Agar	VVM	Polymyxin, colistin, pH (8.6)	Cerdá-Cuéllar *et al*, 2000

* Denotes also selective for *V. cholerae*

1.1.2 Enumeration Methods

There are a number of options available to allow quantitative and semi-quantitative enumeration of *Vibrio* spp.. The first is a direct plating technique using serial decimal dilutions onto microbiological media (i.e. TCBS, CHROMagar-Vibrio). The limit of detection for such methods is generally 10 cfu/g. A Most Probable Number (MPN) technique can also be used. This method is based on the probability of finding bacterial growth after culture of successive dilutions of a food sample in a liquid medium. MPN methodologies are widely used for enumeration of specific organisms or groups of organisms rather than 'total' microbial counts on a product (Roberts & Greenwood, 2003). Alkaline peptone water is usually used as the growth medium for enumeration of *Vibrio* spp. using MPN techniques. Using this method, the level of sensitivity can be set at whatever level is required (practically, the limit of detection is usually set at <0.3 organisms/g but lower levels of detection are possible) by adjusting the dilutions used and referring to standard MPN tables to assign a result.

Whilst this method of detection is more time intensive, it is extremely sensitive as the level of sensitivity is based on the first dilution utilised and detection of even a single cell is theoretically possible within each MPN tube. A combination of MPN and PCR where MPN

tube lysates are used as a DNA template for PCR is considered more sensitive than direct detection by conventional or Real-Time PCR due to the simultaneous examination of thousands of cells in each MPN tube compared to cultural methods that require colony isolation and typically only examine a few suspected colonies (FAO/WHO, 2011).

Both the direct plating and MPN methods require a confirmation step to confirm the presence of virulent species. One such approach is to incorporate molecular methods using an MPN method as a first step prior to the confirmation by PCR or other methods if utilised to directly test samples. Real-Time PCR (refer section 2.6) can also be used to determine the starting level of contamination within the product based on the C_t-value obtained. Sensitivity and accuracy of enumeration from such methodologies will rely on the quality and efficiency of DNA extraction from the sample.

1.1.3 Biochemical Identification

In general, biochemical identification of bacterial isolates is conducted by assessing their ability to metabolise different substrates and/or produce particular enzymes. A practical set of biochemical keys for the routine identification of *Vibrio* spp. known as the Alsina scheme has been developed (Alsina & Blanch, 1994a, 1994b) and has since been updated to reflect increasing species diversity (Noguerola & Blanch, 2008). Other publications also report biochemical identification methods for *Vibrio* spp. (Farmer & Hickman-Brenner, 1992; Garrity *et. al.,* 2004). Biochemical identification is slow and can take up to seven days. However, it is an important set of techniques for identifying presumptive *Vibrio* spp. as it is the only way to identify unknown species that cannot be identified by PCR. Of concern, atypical strains may make identification problematic and it does not generally allow for the determination of likely virulence (the exception being the use of Wagatsuma agar for the detection of tdh expression by *V. parahaemolyticus* – see below). However, the benefits of biochemical identification include the relatively low cost, widespread availability of necessary equipment and the low sensitivity level associated with isolation on selective media.

Commercial miniaturised identification systems are available that may be useful for biochemical identification of *Vibrio* spp. (Anonymous, 2004; Croci *et al*., 2007). These include API 20Ey and API 20NE, which include 20 microtubes with specific media, and Microbact 24E systems, which include 24 microtubes with specific media, that are supplemented with NaCl. Other commercial kits that may be of use are the Crystal E/NF, the MicroScan conventional overnight product, the MicroScan rapid panel, the Vitek GNI+ card, and the Vitek ID-GNB (O'Hara *et al*., 2003). However, a comparative study found that none of the kits achieved an overall accuracy greater than 90% for the identification of *Vibrio* species. The only product to correctly identify at least 90% of *V. cholerae* strains was the Crystal E/NF, and only three of the six products, the API 20E and both of the Vitek cards, correctly identified more than 90% of the *V. parahaemolyticus* strains (O'Hara *et al*., 2003). Furthermore, the accuracy of API 20E test strips to identify *V. parahaemolyticus* can be as low as 42% for environmental isolates and 55% for clinical isolates (Jones *et al*., 2012).

Despite the wide availability of these test kits, poor results have sometimes been encountered, compared with those obtained via classical biochemical approaches (Austin *et al*., 1995). However, there have been few comparative studies undertaken (Noguerola & Blanch, 2008), which makes evaluation of kits versus traditional methods difficult. Moreover, it has been reported that the use of commercial biochemical kits for the identification of environmental isolates could require certain modifications to the manufacturer's protocols as most are

typically intended for clinical use (Noguerola & Blanch, 2008). This can be potentially overcome by the addition of a heavy inoculum and by incubating at 20-21°C for water, fish or shellfish isolates, as well as reading at 24 and 48 hrs (Noguerola & Blanch, 2008).

1.1.4 Serological Identification

Serological identification is based on the use of antibodies to specifically detect antigens/proteins on the bacterial surface. These methods are often used for isolate characterisation (e.g. K and O antigens of *V. parahaemolyticus*), or detection of toxins (e.g. cholera toxin (CT) of *V. cholerae*). More recently, a new method, termed immunomagnetic separation (IMS), has come to prominence as a way of concentrating a particular species or strain of bacteria from a mixed culture, making detection more sensitive. IMS methods have been developed for *V. parahaemolyticus* (Datta *et al*., 2008, Vuddhakul *et al*., 2000) and *V. vulnificus* (Jadeja *et al*., 2010).

The benefits of using serology in the identification of virulent *Vibrio* spp. are mainly around either concentration of the target organism (e.g. IMS), which increases sensitivity when low levels are present, and/or the ability to classify individual isolates (e.g. pandemic *V. parahaemolyticus*). However, these methods generally cannot be used to specifically identify the species of individual isolates as different species often share common antigens. Furthermore, serotypes from sporadic cases and the environment where the product was harvested may not correlate and environmental isolates are frequently untypable (Joseph *et al*., 1982).

1.1.4.1 V. cholerae

Vibrio cholerae can be distinguished serologically on the basis of somatic (O) antigens (Said *et al*., 1994). Over 200 serotypes of this species have so far been identified to date, but only O1 and O139 have been linked to pandemic and epidemic cholera in humans (Cho *et al*., 2010).

Presumptive identification of strains with epidemic potential can be tested using antisera targeting the O antigen of the lipopolysaccharide component of the outer membrane (e.g. serogroups O1 and O139) (Stanley, 2010). Additionally, a commercially available immunoassay has been developed to detect the presence of CT in cultural filtrates of *V. cholerae* and *V. mimicus* (VET-RPLA, Oxoid, Inc., Ogdensburg, NY).

An enzyme-linked immunosorbent assay (ELISA) and a commercial reversed passive latex agglutination (RPLA) kit has also been developed (Said *et al*, 1994, Wong & Desmarchelier, 1999).

1.1.4.2 V. parahaemolyticus

There are very few studies that have attempted to use immunological based methods to identify *V. parahaemolyticus*. Early efforts were directed at the detection of the thermostable direct haemolysin (Honda *et al.,* 1980). However, more recently monoclonal antibodies specific for *V. parahaemolyticus* F_0F_1 ATP synthase have been described (Sakata *et al.,* 2011). The authors found these antibodies to be highly specific for *V. parahaemolyticus* (although some cross-reactivity was found with *V. natriegens*) with VP-Dot technology and hence has been proposed as a possible rapid method for the detection of this organism.

V. parahaemolyticus isolates can also be typed based on their O and K antigens. There are at least 11 O and 70 K antigens that are used to type different *V. parahaemolyticus* isolates (Table 6) (Levin, 2006). The K antigens are surface antigens that are released from the cell by heating to 100°C for 1-2 hours, thus exposing the somatic O antigen. Generally, each K antigen is associated with a single unique somatic O antigen, although there are a few exceptions (Sakazaki *et al*., 1968). As these antigens are common to a range of organisms they are not useful as a front line tool to identify *Vibrio* species. However, once the species is determined isolates can be typed using these antigens.

This information can be used to distinguish what are now known as Pandemic clones of *V. parahaemolyticus*. *V. parahaemolyticus* O3:K6 pandemic clones were first identified during an outbreak of diarrheal cases in Calcutta, India in 1996 (Okura *et al*., 1997). It has since become pandemic in nature, with 14 serovariants discovered (Nair *et al*., 2007).

Table 4: Antigenic scheme of *V. parahaemolyticus*. Adapted from Levin (2006) and Nair *et al*. (2007).

O Group	K Antigen
1	1, 25, 26, 32, 33, 38, 41, 56, 58, 64, 69
2	3, 28
3	4, 5, 6, 7, 29, 30, 31, 33, 37, 43, 45, 48, 54, 57, 58, 59, 65, 75
4	4, 8, 9, 10, 11, 12, 13, 34, 42, 49, 53, 55, 63, 67, 68
5	15, 17, 25, 30, 47, 60, 61, 68
6	18, 46
7	19
8	20, 21, 22, 39, 70
9	23, 44
10	19, 24, 52, 66, 71
11	36, 40, 50, 51, 61

1.1.5 DNA Hybridisation

DNA probes are commonly used to identify virulent *Vibrio* spp. in conjunction with selective media. These methods generally use either a digoxygenin-labelled oligonucleotide probe or an alkaline phosphatase-labelled oligonucleotide probe.

The benefits of this approach include the relatively low cost; they are considered rapid methods and have the ability to detect genes associated with virulence. However, there is a risk that plates used for colony lifts may be overgrown by non-target organisms at low dilutions making quantification potentially problematic.

1.1.5.1 V. cholerae

A digoxygenin-labelled probe can be prepared from PCR amplification product for the detection of the *ctxAB* gene and used for colony hybridisation (Wright *et al*., 1993). The preparation of the probe, the hybridisation conditions for colony blots of suspect isolates and wash protocol follow those outlined in the technical literature provided by the manufacturer (Roche Diagnostics, Indianapolis, IN) (Anonymous, 2004). Digoxygenin-labelled polynucleotide probes CT(A) and CT(B), which can be used to screen non-O1 strains for the presence of the CT gene, have also been described (Said *et al*., 1994). An alkaline phosphatase-conjugated 30-mer oligonucleotide probe has also been developed to specifically

detect the cholera toxin gene (ctx) in *V. cholerae* O1 (Yoh *et al*., 1993). An alkaline phosphatase-labelled oligonucleotide DNA probe specific for *ctx*A has also been described by Wright *et al*. (1992).

1.1.5.2 V. parahaemolyticus

DNA-based probes have been described for the detection of both *tlh* and *tdh* (McCarthy *et al*. 1999 and McCarthy *et al*., 2000 respectively). These have also been adopted by the USFDA Bacteriological Analytical Manual (BAM) as an accepted method for the detection of total and virulent *V. parahaemolyticus* in seafood. The use of DNA-DNA hybridisation for the detection of *V. parahaemolyticus* in seafood and clinical samples using microarray technology has also been developed (Panicker *et al*., 2004, Vora *et al.*, 2005, Wang *et al*., 2011).

1.1.5.3 V. vulnificus

An alkaline phosphatase-labelled DNA probe targeting *vvh* has been described (Wright *et al*., 1993). This method has since been adopted by the USFDA BAM. Since that time, the method has been modified to include a growth step on selective media (VVA) to help limit growth inhibition by competing organisms (DePaola *et al*., 1997). This probe has since been incorporated into a real-time PCR method (Campbell & Wright, 2003) (see Section 2.6). An alternate media and probe combination based on selective growth on mCPC and a probe targeting the 16s rRNA gene has also been described (Cerda-Cuellar *et al*., 2000).

1.1.6 Nucleic Acid Amplification

Nucleic Acid Amplification refers to techniques such as conventional and Real-Time polymerase chain reactions (PCR) that can be conducted as either single or multiplex reactions, as well as Loop-Mediated Isothermal Amplification (LAMP) that can currently only be used as a singleplex reaction. If specific conventional PCR products are amplified, these can be visualised by DNA staining combined with gel electrophoresis, or if a real-time platform is utilised, by fluorescence. DNA amplicons generated by LAMP can be detected either visually in the form of a white precipitate or by measuring the optical density.

DNA amplification methods are becoming increasingly important as a rapid way of identifying potentially contaminated seafood as they can specifically target species-specific and virulence associated genes. The benefits of these approaches are that they are rapid, highly sensitive (even more so when combined with MPN or pre-enrichment as described below) and are highly robust, accurate and repeatable. However, the cost of basic equipment, particularly for Real-Time thermocyclers, may be prohibitive, particularly in developing countries. More recently LAMP has been demonstrated as a low cost method requiring only a heating block or water bath followed by visual inspection for a white precipitate in reaction tubes (pers. comms. M. Nishibuchi) and is comparable to PCR in terms of sensitivity (Jones *et al*., 2012).

A variety of sampling methods can be used when utilising a DNA amplification technique. The most rapid method is to extract DNA directly from a seafood matrix; a technique that has been demonstrated on oysters (e.g. Baker-Austin et al, 2012). However, any DNA extraction technique can result in loss of DNA during processing and sensitivity of the method may also be poor due to low levels of DNA being initially present in the matrix (e.g. Baker-Austin *et*

al. (2012) reported a sensitivity of 7,600 genome copies of the *pilF* gene). To overcome this hurdle, enrichment techniques can be employed to increase sensitivity. Robert-Pillot *et al*. (2010) reported the use of a six hour enrichment of shrimp samples in alkaline peptone water to develop a quantitative assay. However, as mentioned previously, caution needs to be taken when using these methods due to potential differences between samples in the microbial profile that may affect the growth rate of the target organism. Furthermore, assessment of possible inhibition from the matrix also needs to be considered as it may further limit sensitivity.

When evaluating methods for use, it is important to consider how a potential method has been validated (e.g. the number and diversity of strains that the primers have been tested on), whether species identification alone is required or if it is needed in combination with virulence determination. Further consideration must be given as to whether a quantitative or qualitative result is required. However, with correct validation, most PCR techniques can be made semi-quantitative with the addition of either an enrichment step followed by Real-Time PCR detection (keeping in mind the limitations mentioned above) or with the addition of a most probable number (MPN) step which may also improve the sensitivity of a method over direct detection.

There is a wide range of gene targets reported for use as species-specific or virulence markers for *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (genes used to determine potential virulence will be described below). Of concern, many studies report the design of novel oligonucleotide primers and probes for either new locations within previously used genes or previously unutilised sites within the genome rather than using previously reported primers and probes that have been subjected to extensive validation. Consequently, when developing methods for the identification and enumeration of virulent *Vibrio* spp., the selection of an appropriate protocol can be problematic.

1.1.6.1 V. cholerae

The *V. cholerae* genome is made up of a large (chrI) and small (chrII) chromosome. The chrI encodes most of the virulence-related genes whereas chrII encodes several essential metabolic and regulatory pathways (Schoolnik & Yildiz, 2003). Together, these chromosomes accommodate the two distinct *V. cholerae* lifecycles – within the human intestine and long term residence in aquatic habitats subject to climatic variability (Lobitz *et al*., 2000). It is hypothesised that chrI is required for growth and survival in the intestine and chrII for growth and survival in the environment (Schoolnik & Yildiz, 2000).

Genotypic techniques targeting genes from either of these chromosomes are commonly employed to detect and/or identify *V. cholerae* in a range of substrates. A summary of the target genes described in the literature used to identify *V. cholerae* is provided in Table 5. Published methods include conventional single and multiplex PCR, nested PCR, Real Time PCR and LAMP, targeting a diverse group of genes. While the *ctx* gene is commonly used in isolation to identify potentially virulent strains of *V. cholerae* (see below), the most commonly used gene target for the identification of *V. cholerae* is *toxR* (first described by Miler & Mekalanos, 1984). Although it should be noted that the regulatory framework for these pathogens with accepted molecular methodologies to detect *V. cholerae* focus mainly on the virulence-associated factor *ctx*.

Table 5: Target genes used to identify *V. cholerae* described in the available literature

Gene Target	Function	Reference/s*
16S-23S Inter-Spacer Region		Kim *et al*., 2008
*atp*A	Housekeeping gene	Izumiya *et al*., 2011
*dna*J	A house keeping gene encoding heat shock protein 40	Nhung *et al*., 2007
*eps*M	Surface secretion enzyme	Gugliondolo *et al*., 2010
hlyA	Haemolysin	Shangkuan *et al*., 1995
*hsp*60	Heat shock protein	Tebbs *et al*., 2011
*lol*B	Lipoprotein binding protein	Lalitha *et al*., 2008
*omp*W	Outer membrane protein	Nandi *et al*., 2000 Srisuk *et al*., 2010
*pnt*A	Housekeeping gene encoding the transhydrogenase alpha subunit	Teh *et al*., 2010
Flagellin gene	Motility	Hwang *et al*., 2010
*tox*R	Regulatory protein	Bauer & Rorvik, 2007 Ghosh *et al*., 1997 Neogi *et al*., 2010 Panicker *et al*., 2004 Rivera *et al*., 2001
*rec*A	Housekeeping gene	Roozbehani *et al*., 2012
*sod*B	Catalase/peroxidase	Messelhausser *et al*., 2010

* Denotes that only novel oligonucleotide primer sets are referenced

1.1.6.2 V. parahaemolyticus

The *V. parahaemolyticus* RIMD2210633 genome consists of two chromosomes comprising 4832 coding sequences (genes) (Makino *et al*., 2003). A number of these genes have been targeted by PCR for species identification, detection and enumeration from environmental and clinical samples (Table 6). However, the most commonly used targets are *tlh* (first described by Taniguchi *et al*., 1990) and to a lesser extent *toxR* (first described by Lin *et al*., 1993).

Table 6: Target genes used to identify *V. parahaemolyticus* described in the available literature

Gene Target	Function	Reference/s*
16-23S Inter-Spacer Region		Kong *et al*., 2002
*dna*E	Housekeeping gene encoding heat shock protein 40	Tebbs *et al*., 2011
*dna*J	Housekeeping gene encoding heat shock protein 40	Nhung *et al*., 2007
*gyr*B	B subunit of DNA gyrase	Cai *et al*., 2006
*irg*B	Iron regulated virulence regulatory protein	Yu *et al*., 2010
pR72H	Species specific DNA fragment	Lee *et al*., 1995 Robert-Pillot *et al*., 2010 (novel probe only)
tlh	Thermo-labile haemolysin	Bej *et al*., 1999 Nordstrom *et al*., 2007 Oberbeckmann *et al*., 2010
*tox*R	Regulatory protein	Bauer & Rorvik, 2007 Kim *et al*., 1999 Messelhauser *et al*., 2010 Neogi *et al*., 2010

* Denotes that only novel oligonucleotide primer sets are referenced

1.1.6.3 V. vulnificus

V. vulnificus MO6-24/O consists of two chromosomes made up of a total of 4682 coding sequences (genes) (Park *et al*., 2011). Conventional PCR has been used to detect *V. vulnificus* by gene amplification since 1991 (Brauns *et al*., 1991). The gene *vvh* has been the prime target utilised by PCR based assays. More recently however, at least a further six other gene targets have been used for the specific detection of this species (Table 7).

Table 7: Target genes used to identify *V. vulnificus* described in the available literature

Gene Target	Function	Reference/s*
16S rRNA	RNA component of the ribosome	Kim & Jeong, 2001
16-23S Interspacer Region		Kim & Bang, 2008
*dna*J	Housekeeping gene encoding heat shock protein 40	Nhung *et al*, 2007
*rnp*B	Catalytic subunit of RNaseP	Tebbs *et al*., 2011
*rpo*S	Housekeeping gene and general stress regulator	Kim *et al*, 2008
*tox*R	virulence gene regulation	Bauer & Rorvik, 2008
*vvh*A	Haemolysin	Brasher *et al*., 1998 Brauns *et al*., 1991 Campbell & Wright, 2003 Han & Ge, 2008 Hill *et al*., 1991 Lee *et al*., 1998 Messelhauser *et al*., 2010

	Neogi *et al*., 2010

* Denotes that only novel oligonucleotide primer sets are referenced

1.1.7 Gene Sequencing

With the advent of new and more affordable technologies, DNA sequencing is becoming an increasingly useful tool for bacterial identification. Within *Vibrionaceae* a number of genes have been shown to be highly conserved within species. These include 16S rRNA (>97% similarity), *asd* (>96% similarity), *atp*A (>97% similarity), *epd* (>93% similarity), *gap* (>98% similarity), *idh* (>98% similarity), *mdh* (>98% similarity), *obg* (>98% similarity), *rec*A (>95% similarity) and *rpo*A (>99% similarity) (Thompson *et al*., 2004). A subsequent investigation has found some of these genes are also highly conserved between species, including 16S rRNA (up to 99.99% similarity) and recA (up to 97.8% similarity) (Pascual *et al*., 2010). However, there are some genes, such as *toxR*, that do not show any overlap in intra- and inter-species similarity levels (77.2-100% and 33.8-72.5% similarity respectively) that may be more accurate for *Vibrio* species identification as a similarity level will demonstrate that the isolated belongs to either the same species or not (Pascual *et al*., 2010). Therefore, considerable care should be taken when selecting genes for sequencing and subsequent species identification of *Vibrio* spp..

1.1.8 Virulence and Subtyping

Within the natural marine environment, there are potentially both virulent and non-virulent strains of various *Vibrio* spp. This includes those of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Being able to differentiate between the two groups is essential for accurate risk assessment.

A role for flagellum in the virulence of various *Vibrio* spp. has also been discovered. In *V. vulnificus* the flagellum is deemed necessary for virulence in mice. This was a result of decreased adherence to cultured cells, as well as loss of biofilm formation in the absence of flagellum by this bacterium (Lee *et al*., 2004). Furthermore, flagellum-deficient strains were less virulent than the wild type strain when tested using the mouse model. Consequently, it has been proposed that flagella may serve as an adhesin, or as a mediator of invasion of host cells (Lee *et al*., 2004). Indeed, flagella are also required for infection by *V. cholerae*. Non-motile mutants of the El Tor biotype have a 10-fold lower ability to infect cultured mammalian cells compared with the wild type strain (Lee *et al*., 2000).

The virulence mechanisms for *V. cholerae* and *V. parahaemolyticus* have been well characterised (although further investigation is still required, see below) but those for *V. vulnificus* remain elusive. Here we describe some of the methods being employed to discriminate between environmental and potentially virulent *Vibrio* spp..

1.1.8.1 V. cholerae

The virulence of *V. cholerae* O1 (classical and El Tor biotypes) and O139 strains depends on a combination of properties, including the presence of enterotoxin (cholera toxin [CT], *ctxA*) and the ability to adhere to, and colonise, the small intestine (colonisation factor, *tcpA*). These two genes are regulated by the gene *tox*R (Herrington *et al*., 1988).

The major virulence-associated genes are present in at least three clusters. The first is the integrated prophage CTX genetic element that is 7-9 kb in size and known to contain six genes associated with virulence (Waldor & Makalanos, 1996). These include *ctx*AB

(encoding the A and B subunits of CT), *zot* (encoding zonula occludens toxin), *cep* (encoding core encoded pilin), *ace* (encoding accessory cholera enterotoxin), and *orf*lU (encoding a product of unknown function) (Waldor & Makalanos, 1996). The second region is a large virulence island for *V. cholerae* (VPI) that encodes a toxin-coregulated pilus (TCP) gene cluster, a type IV pilus that functions as an essential colonisation factor and acts as a CTX phage receptor. The third gene cluster is the RTX toxin gene cluster which encodes cytotoxic activity for Hep-2 cells in vitro (Rivera *et al*., 2001) based on its actin binding activity. However, the implication of RTX in virulence has yet to be confirmed (DiRita, 2000). The detection of these genes has been described by numerous authors (Table 8).

The Y-1 mouse adrenal cell assay can be applied for the determination of enterotoxigenicity based on the Cholera Toxin (CT) or the *ctx* gene (Anonymous, 2004).

The following are optional tests to further differentiate the Classical from the El Tor biotype (Anonymous, 2004):

A. Beta-haemolysis. The most common means is to determine β-haemolytic ability on sheep blood agar. El Tor strains are β-haemolytic, while classical strains do not produce a hemolysin.

B. Polymyxin-B sensitivity. Classical strains are sensitive whilst El Tor strains are resistant.

Table 8: Genes associated with virulent isolates of *V. cholerae*

Gene	Function	Reference/s*
ctx	Cholera toxin	Blackstone *et al*., 2007 Brasher *et al*., 1998 Espineira *et al*., 2010 Fedio 2007 Koch *et al*., 1995 (BAM) Messelhausser *et al*., 2010 Shangkuan *et al*., 1995
ctxA	Cholera toxin subunit A	Fields *et al*., 1992 Ottaviani *et al*., 2009
Flagellin	Motility and colonisation factor	Hwang *et al*., 2010
hlyA	Haemolysin	Panicker *et al*., 2004 Rivera *et al*., 2001 Shangkuan *et al*., 1995
hlyA (El Tor)	Haemolysin	Ottaviani *et al*., 2009 Rivera *et al*., 2001
stn/sto	Heat stable enterotoxin	Rivera *et al*., 2001
tcpA (Classical)	Colonisation factor	Espineira *et al*., 2010 Ottaviani *et al*., 2009 Rivera *et al*., 2001
tcpI	Colonisation factor	Panicker *et al*., 2004 Ottaviani *et al*., 2009 Rivera *et al*., 2001
toxR	Regulates *ctx*A and *tcp*A	Bauer & Rorvik 2007 Messelhausser *et al*., 2010 Ottaviani *et al*., 2009 Panicker *et al*., 2004 Rivera *et al*., 2001
zot	Zonula occludens toxin	Ottaviani *et al*., 2009

	Rivera *et al*., 2001

* Denotes that only novel oligonucleotide primer sets are referenced

A multiplex single-tube nested PCR (MSTNPCR) assay for *V. cholerae* O1 identification using primers targeted to *ctx*A and *rfb*N genes coding for the cholera toxin and to the O-antigen of *V. cholerae* O1 has also been developed (Mendes *et al*., 2008).

1.1.8.2 V. parahaemolyticus

Disease associated with *V. parahaemolyticus* infections is generally restricted to isolates possessing either the thermostable direct hemolysin (*tdh*) gene, or the thermostable direct hemolysin-related hemolysin (*trh*) gene. A study by Shirai *et al.* (1990) demonstrated that of 285 clinical strains of *V. parahaemolyticus* tested, 52.3% carried the *tdh* gene only, 24.3% carried the *trh* gene only and 11.2% carried both. Only 11% of clinical isolates did not contain either gene. Of 71 environmental strains, only 7% hybridised very weekly with the *trh* gene probe and none hybridised with the *tdh* gene probe. However, a recent study showed that 27% of clinical isolates may lack the *tdh* and *trh* gene suggesting the possibility of 'opportunistic' strains and strains with enhanced virulence potential. These discoveries cast doubt around the reliability of *tdh* and *trh* as predictors of virulence (Jones *et al*., 2012).

The *tdh* and *trh* genes are commonly used to assess for potential virulence using PCR. However, as per species specific gene targets discussed below, there are many novel oligonucleotide primer sets to detect these gene targets by PCR (Bej *et al*., 1991, Blackstone *et al*., 2003, Espineira *et al*., 2010, Messelhauser *et al*., 2010, Nordstrom *et al*., 2007, Tada *et al*., 1992).

The activity of *tdh* may be detected by β-haemolysis on Wagatsuma blood agar, known as the Kanagawa phenomenon (Miyamoto *et al*., 1969). The reproducibility of the Kanagawa phenomenon is dependent on pH, media salinity and erythrocyte type used and as such, identification of virulent serovars by this method is not always accurate (Broberg *et al*., 2011).

More recently, it has been suggested that the presence of Type III Secretion System Type 2 (T3SS2) may be a more reliable indicator of potential virulence (Caburlotto *et al*., 2010). Further, two recent studies have found the presence of the *vopB2* gene of T3SS2 to be more accurate at predicting likely virulence of individual isolates than the presence of *tdh* and/or *trh* (Noriea *et al*., 2010, Jones *et al*., 2012). Such findings suggest more research is needed to fully elucidate the factors common to clinical *V. parahaemolyticus* strains.

1.1.8.3 V. vulnificus

Little is known about the mechanisms of virulence associated with *V. vulnificus* (Moreno & Landgraf, 1998). However, opaque colony morphology has been linked with virulence due to the presence of a capsule (Simpson *et al*., 1987). This was tested by Biosca *et al.* (1993) who demonstrated that reversion to the translucent colony morphology (non-capsulated) resulted in a 1-3 log increase in the LD_{50} of intra-peritoneal injected eels. This was seen to be due to the loss of the protective function of the capsule. Yoshida *et al*. (1985) also observed this effect in mice. These changes in colony morphology, and hence capsular polysaccharide (CPS) expression, are caused by reversible phase variation (Wright *et al*., 2001). The operon responsible for this phenomenon is controlled by an epimerase gene that shares 75 and 85% identity at the nucleotide and amino acid levels respectively with the epimerase of

V. cholerae (Zuppardo & Siebeling, 1998). Apart from this, generic virulence factors are generally investigated to determine potential virulence of *V. vulnificus* isolates. For example, production of proteases, haemolysins and elastase as estimated by biochemical techniques along with *in vitro* cytotoxicity to cultured mammalian cells are usually accepted as indicators of virulence (Levin, 2006).

Determining a specific gene/s to distinguish virulent from non-virulent strains of *V. vulnificus* has so far proven elusive. Recently however, there have been some advancements in this area. Baker-Austin *et al* (2010) designed a conventional PCR to distinguish between clinical (C) and environmental (E) isolates by targeting differences in the *vcg* gene sequences. The *vcg* gene has also been targeted in a method for distinguishing virulent strains of *V. vulnificus* by LAMP (Han *et al*., 2011).

Roig *et al.* (2010) developed another method to distinguish between environmental and clinical isolates based on the *pilF* genes. They then compared their results to those of Rosche *et al.* (2005) and to serum resistance (related to the presence or absence of a capsule). Whilst the serum resistance profiles matched poorly with the results obtained by Rosche *et al*. (2005), those obtained using the Vvpdh oligonucleotide primers were predominantly accurate.

1.2 Considerations for the Selection of an Appropriate Method to Enumerate Pathogenic *Vibrio* Species in Prawns

The need to identify isolates as virulent or not by countries that are developing import and export seafood markets is driven by requirements in the recently developed Codex Committee for Fish and Fishery Products "Guidelines on the Application of General Principles of Food Hygiene to the Control of Pathogenic *Vibrio* Species in Seafood" (Anonymous, 2010). These guidelines were adopted in May, 2011, and suggest that test methods should distinguish between virulent and non-virulent strains. However, the virulence determinants for *V. vulnificus* and to a lesser extent, *V. parahaemolyticus* are currently unclear. The advent of powerful genetic analysis tools such as pyrosequencing (e.g. Morrison *et al*., 2012) will allow for differences in the genomes of clinical and environmental strains of these species, as well as those of *V. cholerae*, to be made clearer in future years. Such analysis would greatly improve our understanding of the differences between virulent and non-virulent strains and allow determination of risk associated with the consumption of various seafood products, including prawns.

When developing and validating methods, there is a need to consider a number of factors. These include, but are not limited to, the level of quantification, limit of detection, whether the selected method can determine potential virulence, robustness, repeatability, time, cost and fitness for purpose. The authors' opinions of these considerations have been summarised for the previously discussed method types and for combinations of different methods where appropriate (Table 9).

The approach required for validation of diagnostic PCR for the detection of food pathogens has been described elsewhere (for a review, see Malorny *et al*., 2003) and so will not be discussed here. However, some important aspects to consider when developing a new PCR-based method are outlined in Table 10.

However, as previously discussed, ISO 21872-1 and ISO 21872-2 are both based on biochemical identification of marine *Vibrio* spp. and are inadequate to meet the Codex guideline. Therefore it is likely that individual jurisdictions develop their methods to meet a

common set of criteria. Further discussion between international jurisdictions will be necessary to agree on what these criteria will be, but those described in Table 11 may provide a starting framework.

1.3 Summary

This review has described methods available in the literature for detecting and enumerating *Vibrio* species (namely *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) in seafood, and to also identify virulent strains. Based on the information provided, it is clear that there are a wide variety of methods described in the literature that may be utilised by agencies responsible for risk assessment. However, a formulated approach that suits individual countries different needs and limitations needs to be adopted.

Table 9: Model criteria for the selection of an appropriate method to detect virulent *Vibrio* spp. in seafood. Those highlighted have been identified as meeting the performance criteria and will be further assessed in Section 2.

	Quantification	Time	Cost	Sensitivity	Virulence	Robustness	Repeatability
ChromAgar	Yes	O/N	Low	Unknown	No	Unknown	High
Singleplex PCR	No	O/N	Low	>100 cfu/g	Yes	Medium	High
Multiplex PCR	No	O/N	Low	>100 cfu/g	Yes	Medium	High
Singleplex Real-Time PCR	Yes	O/N	High	>100 cfu/g	Yes	High	High
Multiplex Real-Time PCR	Yes	O/N	High	>100 cfu/g	Yes	High	High
LAMP	No	O/N	Medium	>100 cfu/g	Yes	High	High
MPN in Selective Broth	Semi	O/N	Low	Unlimited	No	Low	Low
6 hr Enrichment + Real-Time PCR	Semi	O/N	High	1 cfu/25g	Yes	Low	Low
Chromagar + Biochemistry	Yes	1 week	High	10 cfu/g	No	Low	Low
TCBS + PCR	Yes	2 days	Medium	10 cfu/g	Yes	Low	Low
TCBS + Real-Time PCR	Yes	2 days	High	10 cfu/g	Yes	Low	Low
TCBS + Biochemistry	Yes	1 week	High	10 cfu/g	No	Low	Low
Marine Agar + Biochemistry	Yes	1 week	High	10 cfu/g	No	Low	Low
TCBS + Probe	Yes	O/N	Low	10 cfu/g	No	Moderate	Medium
Chromagar + PCR	Yes	2 day	Medium	10 cfu/g	Yes	Low	Low
PCR-ELISA	Yes	O/N	Medium	370 pg DNA	No	Low	Medium
Agar + Probe	**Yes**	**O/N**	**Medium**	**10 cfu/g**	**Yes**	**Moderate**	**High**
MPN + LAMP	**Semi**	**O/N**	**Medium**	**Unlimited**	**Yes**	**High**	**High**
MPN + Standard PCR	**Semi**	**O/N**	**Medium**	**Unlimited**	**Yes**	**High**	**High**
MPN + Real-Time PCR	**Semi**	**O/N**	**High**	**Unlimited**	**Yes**	**High**	**High**

Quantification: refers to whether the method allows for enumeration of the target organism/s in a sample
Time: refers to the time it takes to complete the analysis
Cost: refers to the overall cost of reagents, required equipment and staff time
Sensitivity: refers to the limit of detection
Virulence: refers to whether the method allows for the detection of virulence determinants of the target organism/s
Robustness: refers to the methods ability to remain unaffected by small but deliberate variations in the method parameters (eg. primer and template characteristics, salt concentrations, purity of reagents) and provides an indication of its reliability during normal usage
Repeatability: refers to the methods ability to produce consistent results from a given sample

Table 10: Criteria for standardised diagnostic PCR as per Malorney *et. al*. (2003)

Criteria	Criteria
Analytical and diagnostic accuracy	Low false-negative or false-positive results
Low detection limit	Less than 1 cfu/25 g, particularly for *V. vulnificus*
High robustness (Precision)	Inter-lab reproducibility
Amplification controls	Reagent and positive controls, internal amplification controls
High speed	At-line or on-line analysis
Acceptance	Validation and standardisation, non-patented primer sets
Low cost	
Simplicity	User friendliness and automation
Sample matrix flexibility	No PCR interference
Quantitative analysis	

2 Assessment of Selected Methods for the Identification of Pathogenic *Vibrio* Species

2.1 Introduction

Four methods that scored highly across the key performance criteria have been chosen for further analysis. These are conventional PCR, Real-Time PCR, Loop-Mediated Isothermal Amplification (LAMP) and DNA hybridisation. These methods were chosen due to their potential speed, accuracy, sensitivity, robustness, repeatability and their ability to detect potentially pathogenic variants of the target species (Table 9 – Section 1). These methods are also either being routinely used or are currently under development in internationally recognised laboratories (DNA Hybridisation: USFDA, USA; LAMP: Centre for South East Asian Studies, Japan; Conventional PCR: Centre for Environmental, Fisheries and Aquaculture Science, UK; Real-Time PCR: USFDA, Centre for Environmental, Fisheries and Aquaculture Science, UK)

The aim of this section of the work is to determine the most suitable of these methods for the detection of pathogenic vibrios in prawns by trialing these methods within our laboratory to fully assess their suitability.

2.2 Chosen Methods

The methods selected for initial assessment were conventional PCR, Real-Time PCR, LAMP and DNA hybridisation. All methods were assessed for robustness and repeatability by assessing their accuracy under optimal and sub-optimal annealing temperatures, Mg^{2+} concentration and primer concentration in the reaction mix.

The cultures used for this work were:

1. *V. cholerae* O1 (*ctx*$^+$) DNA provided by Melbourne Diagnostic Unit (MDU).
2. *V. parahaemolyticus* 2341 (tdh$^+$/trh$^+$ pandemic clone) provided by the Centre for South East Asian Studies (CSEAS), Japan.
3. *V. vulnificus* FS12-0344 provided by MDU.

These isolates were maintained on marine agar slopes [peptone (1%), yeast extract (1%, w/v), NaCl (3%, w/v) and agar (0.5%, w/v)] at room temperature. Cultures were then streaked for single colonies on marine agar with a single colony subsequently cultured in alkaline peptone water (APW) [peptone (2%, w/v), NaCl (2%, w/v), pH 8.6±0.2] overnight at 37°C as required.

Crude DNA lysates were prepared for LAMP, conventional PCR and Real-Time PCR based on the procedure described by Blackstone *et al*. (2003). Briefly, a bacterial suspension (1 mL) grown overnight in APW as above was boiled for 5 min and immediately placed on ice for 10 min. Lysates were then centrifuged at 14,000 × RPM for 10 minutes. The supernatant was then aliquoted (10 µL) into eppendorf tubes and stored at -18°C until use (i.e. all tubes had the same treatment for all reactions). Tubes were then thawed and the suspended DNA diluted 1:100 with sterile distilled water. All aliquots were frozen and defrosted as required.

2.2.1 DNA Hybridisation

DNA Hybridisation of the *V. parahaemolyticus* specific *tlh* gene was assessed as per the Bacteriological Analytical Manual (Anonymous, 2004). Alkaline-Phosphatase (AP) probes were initially made using an oligonucleotide probe from Geneworks (Adelaide, Australia) that was subsequently labeled with AP using a Biotin Chromagenic Detection Kit (Thermo Scientific, Vilnius, Lithuania) as per manufacturer's instructions. Subsequent AP-labeled probes were sourced from DNA Technology AS (Risskov, Denmark) as they are the only known supplier worldwide of AP-labelled oligonucleotide probes. Samples were prepared by alternately spot plating *V. parahaemolyticus* and *V. vulnificus* onto a marine agar plate and grown overnight at 37°C. Colony lifts and hybridisations were performed as per the described method.

2.2.2 LAMP

A commercial kit (Eiken Chemical Co. Ltd.) was used for LAMP analysis. Therefore, $MgSO_4$ concentration could not be adjusted. Consequently, only temperature (±2°C from the published method) and primer concentration (±0.5 µM from the published method) were assessed for their effect on robustness. Reactions were run using a Maxygene Gradient Thermal Cycler (Axygen, Union City California) and results visualised by eye. All LAMP reactions were conducted in duplicate.

2.2.2.1 V. parahaemolyticus

Total *V. parahaemolyticus* was detected by LAMP as per Nemoto *et al*., 2011 (Table 11). *Tdh* and *trh* (only *trh*1 was assessed as this is the gene present in the positive control organism) were detected by LAMP as per Yamazaki *et al*., 2010 (Table 11).

2.2.2.2 V. vulnificus

V. vulnificus was detected by LAMP as per Han & Ge, 2008 (Table 11).

Table 11: Oligonucleotide primers and reaction conditions for the detection of *tlh*, *tdh*, *trh*1, *trh*2 and *vvh* genes by loop-mediated amplification

Gene Target	Oligonucleotide Primers	Reaction Temperature	Reference
tlh	F3: catcggcaccaaatttctacttg B3: gaacggttttaacaaactctggag FIP: cgccaacatcaggagtatacggacgctgaccaacaaaagcgg BIP: gcggagagacacagagtagaaatcgttgagtcag LF: gcttcctaatcgtacaacttc	65°C, 60 min	Nemoto *et al*., 2011
tdh	F3:agatattgtttgttgttcgagat B3: aacacagcagaatgaccg FIP: gtacctgacgttgtgaatactgattgtctctgacttttggacaaac BIP: tgacatcctacatgactgtgaacacttatagccagacaccgc LF: gtacggttttctttttacattacg LB: aagactatacaatggcagcg	65°C, 60 min	Yamazaki *et al*., 2010
*trh*1	F3: gcgcctatatgacggtaa B3: acattgacgaaatattctggc FIP: aggcttgtttttctgattttgtgactacacaatggctgctct BIP: tcttctgttagtgatttcgttggttttcatccaatacgttacact LF: agaccgttgaraggcc	65°C, 60 min	Yamazaki *et al*., 2010
*trh*2	F3: catcaatacctttttccttctcc B3: gcttgttttctctgattttgtg FIP: ccgattgaccgtatacatctttgttgtggaggactattggacaa BIP: tcaaagtggttaagcgcctatatgccatstttataaccagaaagagc LF: tggttttctttttatgkttcggt LB: atggtcayaactatacratggc	65°C, 60 min	Yamazaki *et al*., 2010
vvh	F3: cggcaacgtcagatggtt B3: cgggctttttcggtgtgta FIP: atgaatacccgtgccaggctttcctgacgccaaaattgtcc BIP: cacagctggttccagagttgggcgggtttcacccaaaggt	63°C, 60 min	Han & Ge, 2008

2.2.3 Conventional PCR

Reaction mixes and cycling conditions were as per the appropriate referenced method below. All reactions contained the required volume of dNTP's (Invitrogen, Carlsbad California), Platinum Taq (Invitrogen), 1 × Rxn Buffer and Mg^{2+} as described in the original methods. Oligonucleotide primers were obtained from either Geneworks or IDT (Coralville, Iowa).

Reactions were run using a Maxygene Gradient Thermal Cycler. All PCR reactions were conducted in duplicate visualised by gel electrophoresis. Electrophoretic separation of DNA fragments was carried out at room temperature using 1% (w/v) agarose gels prepared in 1 × Tris-acetate-EDTA (TAE) buffer (50 × TAE Buffer: 242 g Tris base, 57.1 mL Acetic acid, 100 mL 0.5 M EDTA, ddH2O to 1 litre and adjusted pH to 8.5) containing 1 × GelRed DNA stain (Biotium, Hayward California). A Hyperladder IV DNA ladder (Bioline, London England) was used as molecular weight markers for DNA fragments separated in agarose gels. Gels were run at 100 V in 1 × TAE buffer. Gels were visualised using a transilluminator and photographed using an E-Box VX2 (Vilber Lourmat, France).

2.2.3.1 V. cholerae

V. cholerae was detected by conventional PCR at the species/pathogenicity level (*ctx* gene)as per Koch *et al.*, 1993 (Table 12). Three annealing temperatures (53°C, 55°C and 57°C), Mg^{2+}

concentration (0.5 mM, 1.5 mM and 2.5 mM) and primer concentration (0.1 mM, 0.5 mM and 1 mM) were assessed in combination.

2.2.3.2 V. parahaemolyticus

V. parahaemolyticus was detected by conventional PCR at the species (*tlh* gene) and pathogenicity level (*tdh* and *trh* genes) as per Bej *et al*., 1999 (Table 12). Three annealing temperatures (56°C, 58°C and 60°C), Mg^{2+} concentration (2 mM, 3 mM and 4 mM) and primer concentration (0.1 mM, 0.5 mM and 1 mM) were assessed in combination.

2.2.3.3 V. vulnificus

V. vulnificus was detected by conventional PCR (species level only, *vvh* gene) as per Brauns *et al*., 1991 with minor alterations (Table 12). Three different annealing temperatures (60°C, 62°C and 64°C), Mg^{2+} concentration (2 mM, 3 mM and 4 mM) and primer concentration (0.1 mM, 0.5 mM and 1 mM) were assessed in combination.

Table 12: Oligonucleotide primers and reaction conditions for the detection of *ctx*, *tlh*, *tdh*, *trh* and *vvh* genes by conventional PCR

Gene Target	Reference	Oligonucleotide Primers	Cycling Conditions*
ctx	Koch *et al*., 1993	P1 5'-tgaaataaagcagtcaggtg-3' P2 5'-ggtattctgcacacaaatcag-3'	94°C 3 min, 30 cycles of 94°C 1 min, 53°C, 55°C* or 57°C 1 min, 72°C 1 min
tlh	Bej *et al*., 1999	L-tl: 5'-aaagcggattatgcagaagcactg-3' R-tl: 5'-gctactttctagcattttctctgc-3'	94°C 3 min, 30 cycles of 94°C 1 min, 56°C, 58°C* or 60°C 1 min, 72°C 1 min
tdh	Bej *et al*., 1999	L-tdh: 5'-gtaaaggtctctgacttttggac-3' R-tdh: 5'-tggaatagaaccttcatcttcacc-3'	94°C 3 min, 30 cycles of 94°C 1 min, 56°C, 58°C* or 60°C 1 min, 72°C 1 min
trh	Bej *et al*., 1999	L-trh: 5'-ttggcttcgatattttcagtatct-3' R-trh: 5'-cataacaaacatatgcccatttccg-3'	94°C 3 min, 30 cycles of 94°C 1 min, 56°C, 58°C* or 60°C 1 min, 72°C 1 min
vvh	Brauns *et al*, 1991	Vv 1: 5'-cgccgctcactggggcagtggctg-3' Vv 3: 5'-ccagcgttaaccgaaccacccgc-3'	94°C 3 min, 30 cycles of 94°C 1 min, 63°C, 65°C* or 67°C 1 min, 72°C 1 min

2.2.4 Real-Time PCR

All reactions contained the appropriate volume of dNTP's, Platinum Taq, 1 × Rxn Buffer and Mg^{2+} as described in the original methods below. All Real-Time PCR methods were assessed for robustness and linearity. Robustness was assessed by altering the annealing temperature ±3°C and determining the effect on C_t values. Linearity was assessed by using serial decimal dilutions of prepared DNA template in the reaction mix with the resultant C_t values plotted against the dilution factor. Linear regression was determined using the Rotorgene Q Series Software program. All reactions were run using a Rotor-Gene Q (QIAGEN, Düsseldorf, Germany). Oligonucleotide primers were sourced from GeneWorks and the oligonucleotide probes from either IDT (BHQ-2-based probes) or Life Technologies (MGB-based probes).

2.2.4.1 V. cholerae

V. cholerae was detected by Real-Time PCR as per Blackstone *et al*., 2007 (Table 13).

2.2.4.2 V. parahaemolyticus

V. parahaemolyticus tlh, *tdh* and trh genes were detected by Real-Time PCR as per Nordstrom *et al*., 2007 with some alterations (Table 13). Specifically, individual gene targets were assessed in a duplex reaction with the internal amplification control (IAC) only. These duplex methods were developed by USFDA but remain unpublished (pers. comms. Jessica Jones, USFDA).

2.2.4.3 V. vulnificus

V. vulnificus was detected by Real-Time PCR as per Jones *et al*., 2009 (Table 13).

Table 13: Oligonucleotide primers and reaction conditions for the detection of *ctx*, *tlh*, *tdh*, *trh* and *vvh* genes by Real-Time PCR

Gene Target	Reference	Oligonucleotide Primers and Probes	Cycling Conditions*
ctx	Blackstone *et al*., 2007	Sense: 5′-tttgttaggcacgatgatggat-3′ Anti-Sense: 5′-accagacaatatagtttgacccactaag-3′ Probe: FAM-tgtttccacctcaattagtttgagaagtgccc-BHQ	94 °C for 2 min, followed by 45 cycles 94°C for 10 s, 61°C, 63°C* or 65°C for 30 s
tlh	Nordstom *et al*., 2007	*tlh* f: 5'-actcaacacaataatatatcgacaa-3' *tlh* r: 5'-gatgagcggttgatgtccaa-3' *tlh* probe: 5'FAM-cgctcgcgttcacgaaaccgt-3'BHQ2 IAC f: 5'-gacatcgatatgggtgccg-3' IAC r: cgagacgatgcagccattc-3' IAC probe: 5'Cy5-tctcatgcgtctccctggtgaatgtg-3'BHQ2	45 cycles of 95°C for 5 s 59°C for 45 s
tdh	Nordstom *et al*., 2007	*tdh* f: 5'-tccctttttcctgccccc-3' *tdh* r: 5'-cgctgccattgtatagtctttatc-3' *tdh* probe: 5'FAM-tgacatcctacatgactgtg-3'MGBNFQ IAC as per *tlh*	45 cycles of 95°C for 5 s 59°C for 45 s
trh	Nordstom *et al*., 2007	*trh* f: 5'-ttgctttcagtttgctattggct-3' *trh* r: 5'-tgtttaccgtcatataggcgctt-3' *trh* probe: 5'FAM-agaatacaacaatcaaaactga-3'MGBNFQ IAC as per *tlh*	45 cycles of 95°C for 5 s 59°C for 45 s
vvh	Jones *et al*., 2009	Forward: 5'-tgtttatggtgagaacggtgaca-3' Reverse: 5'-ttctttatctaggccccaaacttg-3' Probe: 5'FAM-ccgttaaccgaaccacccgcaa-3'TAMARA IAC: as per *tlh*	45 cycles of 95°C for 10 s 60°C for 45 s

2.3 Results and Discussion

Results tables for each method where appropriate are provided in Appendix 1.

2.3.1 DNA Hybridisation

DNA hybridisation was assessed as per the Bacteriological Analytical Manual. Initial attempts at labeling the DNA probe using the Biotin Chromagenic Detection Kit were unsuccessful. It was later found that AP-labeled oligonucleotide probes could be obtained from a single supplier, located in Denmark (pers. comms. Jessica Jones, USFDA). It took a significant amount of time to optimise the method for use in our laboratory using these probes and even then in our hands the results were inconsistent, with mis-labeled

V. vulnificus colonies by the *tlh* probe and poor labeling of *V. parahaemolyticus* common problems. Consequently, due to difficulties establishing the method and inconsistent results achieved with the method once established, only the method for *tlh* (*V. parahaemolyticus*) was assessed.

This method requires selecting the correct colonies from TCBS plates, which may be overgrown with other non-target *Vibrio* spp. or be difficult to distinguish. It is our experience that there are many *Vibrio* species that appear similar to *V. parahaemolyticus* or *V. vulnificus* on TCBS agar. DNA hybridisation was also a labour intensive method, with a full day required to conduct the DNA hybridisation step. Overall, the method took four days to process an individual sample, compared to a day and half for Real-Time PCR, and two for conventional PCR. Based on the initial difficulties establishing the method and also on the time to complete the analysis, it was considered that the method was not suitable for use as a method for detecting and enumerating pathogenic vibrios in prawns.

2.3.2 LAMP

The LAMP method for detection of *tdh* and *trh* as per Yamazaki *et al*. (2010) was effective at the proscribed annealing temperature and primer concentrations. This method was not improved when these parameters were slightly altered. A change in primer concentration in particular caused the reaction to fail. This was also the case for *tlh* as per Nemoto *et al*. (2011). The method for *vvh* as per Han and Ge (2008) gave very poor results in our hands regardless of the primer concentration and annealing temperature. Very few positive results were achieved with additional difficulties in determining positive results by eye (as opposed to a turbidity reader). The method for *ctx* was not assessed as it was clear from these results that LAMP was not suitable for all target species, particularly in the absence of a turbiditimeter. All results are provided in Tables A1.1 – A1.4.

LAMP is a relatively new technology and there is currently only one supplier of the reagents worldwide (Eiken Chemical Co. Ltd.). The key advantage of using LAMP is that only a heating block is required, although most methods include the use of a turbiditimeter built into the heating block so that measurements can be taken that allow for enumeration, similar to Real-Time PCR. Indeed, these results may have been improved with access to the specialised Loop-amp Real-Time Tubidimeter. A loan Loopamp real-time turbiditimeter was requested from Eiken Chemical Co. Ltd but it was not possible to fulfill this request. The cost of a unit (~$40,000) also exceeded the operating budget for this project.

The reaction mixes for LAMP assays are currently only available from Eiken Chemical Co. Ltd. Furthermore, in the absence of a turbidimeter (as in our case), determining positive/negative reaction tubes could at times be difficult. No internal amplification control is possible with this method meaning that it is not possible to detect inhibition from the sample. Therefore, it is difficult to be truly confident that a negative result is the consequence of a negative sample. However, with further research and increased acceptance and use, the cost and ease of use is expected to be greatly reduced. Furthermore, it is envisaged that in the future the reagents may be freeze-dried allowing storage at room temperature and easy shipping. However, the absence of an internal control is unlikely to be easily overcome without using duplicate tubes for every sample.

Whilst the method provides a positive or negative result by eye alone, quantification could also be implemented by use of an MPN method, meaning a turbiditimeter would not be required, making it an even more affordable option. With only a heating block required, this

approach could potentially be very attractive to research and food safety bodies with small budgets, as the method is also being applied to an increasing number of food pathogens (e.g. *Campylobacter* (Yamazaki, 2013), enterotoxigenic *Escherichia coli* (Jiang *et. al., 2*012), *Listeria monocytogenes* (Wang *et. al*., 2011)). However, as evidenced with the difficulties mentioned above, further development is required before it can be reliably utilised.

2.3.3 Conventional PCR

Conventional PCR was assessed as per Koch *et al*. (1993) (*ctx*), Bej *et al*. (1999) (*tlh, tdh* and *trh*) and Brauns *et al*. (1991) (*vvh*). Conventional PCR was found to be robust for all gene targets at the published oligonucleotide primer/Mg^{2+} concentration and annealing temperature. The greatest impacts were seen with sub-optimal Mg^{2+} for *V. cholerae*, decreased annealing temperature for *V. parahaemolyticus* and sub-optimal Mg^{2+} or increased oligonucleotide primer concentration for *V. vulnificus*. These resulted in either non-specific amplification or no amplification at all. An example of a typical result is shown in Figure 1. All results are provided in Tables A1.5 – A1.7.

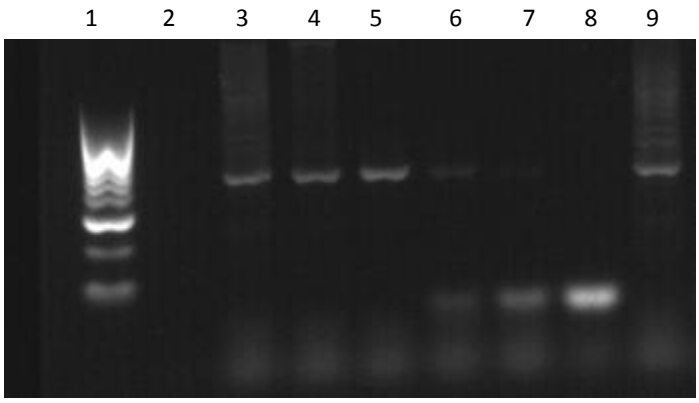

Figure 1: Typical Conventional PCR result for *tlh*.

1. DNA size marker
2. Negative control
3. Smear caused by inefficient amplification
4. Good amplification
5. Good amplification
6. Weak amplification
7. No amplification
8. No amplification
9. Smear caused by ineffective amplification

There are several inherent problems with conventional PCR methodologies that are difficult to resolve. These include there being no published methods that include an internal amplification control meaning that it is not possible to detect inhibition from the sample. Whilst multiplex reactions including an internal amplification control can be designed, such methods can be difficult to optimise in conventional PCR reactions. Therefore, as with LAMP, it is difficult to be truly confident that a negative result is the consequence of a negative sample. While this method is cheaper than Real-Time PCR, it requires a longer cycling time followed by loading and running an agarose gel and requires the use of potentially harmful reagents (e.g. ethidium bromide). While staining alternatives are available, there are still questions regarding their long-term safety.

2.3.4 Real-Time PCR

Some optimisation from the USFDA in-house methods was required. In particular, the described method for *tlh*, *tdh* and *trh* all used Texas-Red (*tlh*, *tdh* and *tdh* probes) and Cy5 (IAC probe) fluorophores. This resulted in cross-talk between the acquiring channels when using the RotorGene Q platform. Consequently, the *tlh*, *tdh* and *trh* probes were altered to include FAM instead of Texas-Red. With these adjustments, the Real-Time PCR methods assessed were found to provide highly linear results with serial decimal dilutions of the target DNA with the r^2 values of 0.9865 (ctx), 0.9976 (*tlh*) and 0.9869 (*vvh*) at the proscribed reaction concentrations (an example is provided in Figure 2). Efficiencies were 0.63, 0.88 and 0.85 respectively. No changes in C_t values were observed with changes in annealing temperature, suggesting the methods were robust.

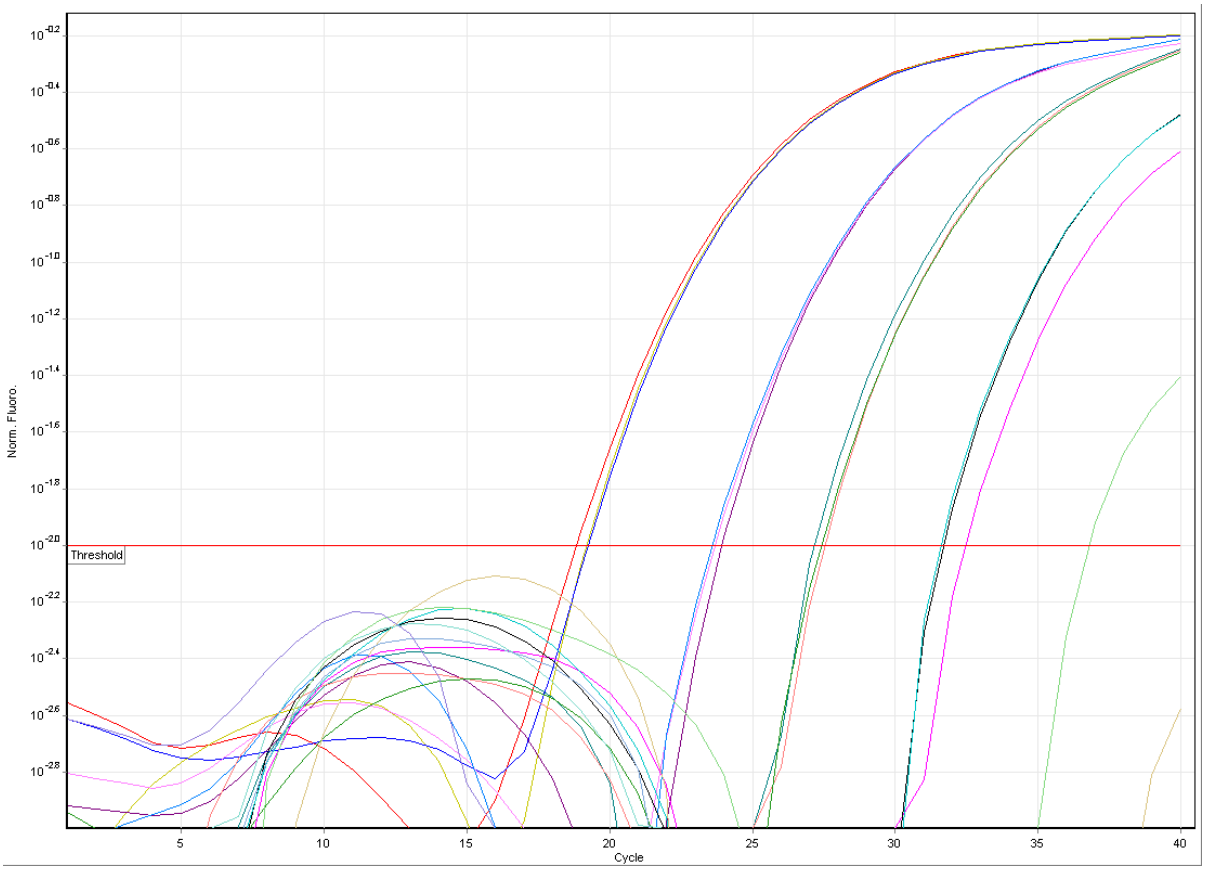

Figure 2: Example of Real-Time PCR results achieved using serial decimal dilutions of target DNA. This example shows the results obtained for *tlh* using serial decimal dilutions of *V. parahaemolyticus* DNA template

The inclusion of an IAC in every reaction provides confidence in negative results and no potentially harmful chemicals are required, as with conventional PCR. It should be noted that for *ctx* and *vvh* reactions that inhibition of the IAC was noted in *ctx* or *vvh* positive samples. This is in line with results achieved at USFDA (pers. comms. Jessica Jones) and is only present in the positive samples. Therefore, it is still an effective IAC.

Real-Time PCR is also a very rapid method requiring only a day and half from sample collection to result, with enumeration possible using an MPN method. In our hands these methods were robust with changes to annealing temperature and Mg^{2+} concentration. It also

has the advantage of including an oligonucleotide probe, meaning it is potentially more specific than conventional PCR, although LAMP uses up to six oligonucleotide primers, suggesting that this would enhance specificity even further.

2.4 Summary and Conclusion

Based on the review of methodologies undertaken in Section 1, four methods for detecting these pathogens were chosen for further development (conventional PCR, Real-Time PCR, Loop-Mediated Isothermal Amplification (LAMP) and DNA hybridisation). All of these methods have been described in the literature as suitable for this purpose but all have their own pros and cons, as evidenced by our results.

The main considerations when selecting a method to move to the validation stage for our purposes were specificity, robustness, repeatability and speed. Based on these findings (summarised in Table 14), Real-Time PCR achieved the highest score on all considerations and also included an internal amplification control (IAC). The IAC provides the additional safety of ensuring there is no inhibition of amplification from the sample, a common problem with shellfish. However, it is important to note that Real-Time PCR is also the most costly due to the cost required for instrumentation and reagents required. Specialist skills are also required to trouble-shoot and analyse the results. Therefore, it may not be the most suitable for all laboratories. Nevertheless, it was decided as the most appropriate method and the decision made to proceed with Real-Time PCR for the final stages of the project.

Table 14: Summary of considerations of each method trialled for this project.

Method	Time	Specificity	Robustness	Cost	Safety	Repeatability
LAMP	1.5 d	High	Low*	Moderate	High	Low*
DNA Hybridisation	4 d	Low**	Low**	Low	High	Low
Conventional PCR	2 d	Moderate	Moderate	Low	Moderate	High
Real-Time PCR	1.5 d	High	High	High	High	High

* Refers that LAMP was unreliable when reaction conditions were slightly altered for all gene targets assessed and poor results were obtained for *vvh* (*V. vulnificus*) even with the described reaction conditions.

** Refers DNA Hybridisation resulted in false positive results from *V. vulnificus* when the *tlh* (*V. parahaemolyticus*) gene probe was used and false negative results from *V. parahaemolyticus* from the same probe.

3 Validation of Real-Time PCR for the Detection and Enumeration of Pathogenic *Vibrio* Species in Australian Prawns

3.1 Introduction

Section 2 described the assessment of four different methods for the detection and enumeration of pathogenic *Vibrio* species in prawns. Based on the results obtained and the criteria set in Section 1, Real-Time PCR was selected as the most appropriate for the purposes of detection and enumeration of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in Australian prawns. Although it was the most expensive method trialed, Real-Time PCR proved to be highly valuable for its high speed, specificity, robustness, safety and repeatability.

The aims of this section were as follows:

1. Develop a *Vibrio* isolate library for use to further assess the specificity of the Real-Time PCR methods using local and international *Vibrio* isolates of the target and non-target *Vibrio* species.
2. Assess CHROMagar as an alternative to TCBS for periodical independent checks of positive MPN tubes.
3. Assess the ability of Real-Time PCR to detect and enumerate pathogenic *Vibrio* spp. in spiked and natural samples.

3.2 Methods

3.2.1 *Vibrio* Library

A library of various *Vibrio* strains from Australia and International sources was developed. A call for strains was sent to a number of Australian and international research institutes known to be working with vibrios. DNA samples of *V. cholerae* were also sought due to state and federal legislation restrictions against storing live cultures of this organism. Other *Vibrio* isolates were also obtained from a number of food sources.

To develop a variety of Australian strains for inclusion in the library, a number of seafood products were obtained from around Australia (Table 15). Samples (25 g) were diluted 1:10 with peptone saline water. Aliquots (100 µL) of serial decimal dilutions were then grown on TCBS agar overnight at 30°C. The dilution showing 30-300 isolates was then selected for each sample. From these plates, five random isolates were streaked for single colonies onto marine agar and grown overnight at 30°C. A single isolate was then inoculated into saline alkaline peptone water (APW) and again incubated overnight at 30°C. An aliquot (100 µL) of this culture was subsequently spread onto marine agar and grown overnight at 30°C to produce a lawn plate, from which 2-3 loops-full of growth were inoculated into snap-freeze medium (Oxoid, Adelaide, South Australia) and stored at -80°C until use. Individual isolates were then identified using the Alsina scheme (Noguerola & Blanch, 2008). Briefly, Gram-negative, oxidase positive, motile rods that have been isolated on TCBS agar are grouped according to their ability to produce arginine dihydrolase (A), lysine decarboxylase (L) and ornithine decarboxylase (O). The isolates are then assessed by a battery of up to 14 sequential biochemical tests (e.g. production of indole, Voges Proskauer test, citrate production) depending on their A/L/O profile to provide a final identification. Suspected

V. parahaemolyticus isolates, including presence or absence of the *tdh* and *trh* genes, were confirmed as per Nordstrom *et al*. (2007) and potential *V. vulnificus* isolates as per Jones *et al*. (2009).

Table 15: Australian seafood products used to obtain *Vibrio* isolates for the *Vibrio* strain library

Product	Harvest Date
NSW Southern Rock Oyster	May, 2011
SA Pacific Oyster	December, 2011
Tasmanian Pacific Oyster	February, 2010
East Coast Blood Pipis	October, 2011
School Prawns	October, 2011

3.2.2 *Vibrio* CHROMagar as a Method to Distinguish Environmental and Clinical *V. parahaemolyticus* and *V. vulnificus* from environmental *Vibrio* Species

As part of standard practice, it is recommended to periodically streak positive MPN tubes onto a selective agar to ensure the presence/absence of the target organism to confirm the PCR results. This confirmatory action is taken as there is potential that target genes may be located in non-target *Vibrio* genomes. Isolates are usually selected from TCBS agar, however *Vibrio* CHROMagar has been suggested to be more discriminatory for the identification of *V. cholerae* and *V. vulnificus* (aqua colonies) and *V. parahaemolyticus* (mauve colonies) (Hara-Kudo *et al*., 2001). Therefore, to assess the accuracy of *Vibrio* CHROMagar in the Australian situation, 106 random isolates were streaked for single colonies on *Vibrio* CHROMagar (Springfield, USA). The presence/absence of growth and colony colour following overnight incubation at 30°C was recorded.

3.2.3 Assessment of *Vibrio* Library Using Real-Time PCR

The five different Real-Time PCR methods (*ctx* as per Blackstone *et al* (2011), *tlh*, *tdh* and *trh* as per Nordstrom *et al*. (2007) and *vvh* as per Jones *et al* (2009)) were assessed for their accuracy against a selection of 101 *Vibrio* isolates from the *Vibrio* library. These are presented in Table 10. The methods were also assessed for false positive results against eight non-*Vibrio* isolates. Crude DNA extracts for each isolate were prepared as per Section 2. 1:100 dilutions of the lysates were prepared in sterile water of which 2 µL was used as DNA template in each Real-Time PCR. All isolates were assessed by each method in duplicate PCR runs.

Table 9: List of isolates used to assess the accuracy of Real-Time PCR for the identification of virulent *V. cholerae*, *V. parahaemolyticus* (including virulence associated genes) and *V. vulnificus*.

Gene Target	*V. cholerae* (*ctx*$^+$)	*V. parahaemolyticus* (*tdh*$^+$/*trh*$^+$)	*V. vulnificus*	Other *Vibrio* Species	Non-*Vibrio* Species
ctx	5 (3)	36 (10/7)	3	58	8
tlh	5 (3)	36 (10/7)	3	58	8
tdh	5 (3)	36 (10/7)	3	58	8
trh	5 (3)	36 (10/7)	3	58	8
vvh	5 (3)	36 (10/7)	3	58	8

3.2.4 Assessment of Real-Time PCR to Detect Pathogenic *Vibrio* Species in Mixed Culture Comprising Multiple *Vibrio* Species

Twenty mixed cultures were produced by inoculating individual tubes containing APW (10 mL) with *V. parahaemolyticus* 2341, *V. vulnificus* FS12-0344, *V. fluvialis* (MDU), *V. alginolyticus* FS11-2570 and FS12-0343, *V. gigantis* FS11-2194.1 and *V. mimicus* (MDU). The mixture was then grown overnight at 30°C. One tube was grown without the addition of *V. parahaemolyticus* or *V. vulnificus* as a negative control. Crude DNA substrate was then made as per Section 2. 1:100 dilutions of the lysates were prepared in sterile water of which 2 µL was used as DNA template in each Real-Time PCR. Real-Time PCR was then used to detect *V. parahaemolyticus* and *V. vulnificus* in the sample as per the described method. Given the restrictions on culturing *V. cholerae* O1/O139, *ctx* was assessed by adding 1 µL of the mixed culture DNA lysates and 1 µL of the *V. cholerae* O1 DNA lysates to each Real-Time PCR reaction mix. All mixed cultures were assessed for each gene target in duplicate.

3.2.5 Assessment of Inoculated Prawn Samples by Real-Time PCR

Inoculated prawns were used to determine each method's capability of enumerating the target *Vibrio* species at low levels with respect to traditional plating methods. A suspension of *V. parahaemolyticus* (*tdh*$^+$/*trh*$^+$ strain) or *V. vulnificus* was prepared in saline APW with growth overnight at 37°C. The method for *V. cholerae* could not be assessed in this manner as only specialised legally accredited laboratories are permitted to culture *V. cholerae* O1/O139 and we were unable to source a *ctx*$^+$ *V. cholerae* nonO1/O139 isolate. Serial decimal dilutions were made in APW and 1 mL equating to ~250, ~2500, ~25,000 and ~250,000 cells was added to 25 g of prawn meat prepared as per AS5013.18-2010 (equating to ~10, ~100, ~1,000 and ~10,000 cfu/g of prawn meat). Each target was assessed in triplicate. Saline APW (224 mL) was then added and the suspension stomached for 1 min. MPN tubes were then prepared as per AS5013.18-2010. Briefly, aliquots (1 mL) of serial decimal dilutions of the homogenate were added to saline APW in triplicate. A negative control for each batch was included with no inoculum added to the prawn sample. The MPN tubes were then grown overnight at 37°C. Crude DNA lysates were prepared from positive tubes for Real-Time PCR by boiling an aliquot (1 mL) from each positive tube for 5 min and immediately placing on ice for 10 min. Lysates were then centrifuged at 14,000 × RPM for 10 minutes. The supernatant was then aliquoted (10 µL) into eppendorf tubes and stored at -18°C until use. Tubes were then thawed and the suspended DNA diluted 1:100 with sterile distilled water for use. Initial inoculum levels were determined by plating the serial decimal dilutions of the inoculum onto TCBS agar, incubating them overnight at 37°C and counting the colonies typical of the respective *Vibrio* species.

Prepared DNA lysates were then assessed as per the optimised Real-Time PCR for *V. parahaemolyticus* or *V. vulnificus* outlined above as appropriate. Each PCR was conducted in duplicate.

3.2.6 Australian Prawn Survey

A small survey of raw Australian prawns was conducted using samples collected as per the Cadmium Survey that was also undertaken as part of Seafood CRC Project: 2009/787. Twenty nine random prawn samples collected for the Cadmium Survey were screened for the presence of pathogenic *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* using the

optimised duplex methods. The presence/absence of *tdh* and *trh* was only determined in *V. parahaemolyticus* positive tubes.

3.3 Results and Discussion

3.3.1 *Vibrio* Culture Collection

A culture collection containing 196 individual *Vibrio* isolates has been stored in snap freeze medium at -80°C along with five *V. cholerae* boiled DNA lysates. These include isolates from the Australian Institute of Marine Science (six isolates), ATCC (38), DSMZ (German Culture Collection, 10 isolates), the Centre for Environment, Fisheries and Aquaculture Science, UK (20 isolates), the Centre for South east Asian Studies, Japan (six isolates including one pandemic *V. parahaemolyticus*) and the Melbourne Diagnostic Unit (eight isolates and five *V. cholerae* boiled DNA lysates). A full list of isolates within the culture collection is presented in Appendix 2.

This *Vibrio* strain library contains isolates from both local and international sources. Such a resource is critical for appropriate assessment of methodologies and will greatly enhance/underpin future research into these organisms in Australia.

3.3.2 Assessment of CHROMagar

One hundred and six random isolates were grown on *Vibrio* CHROMagar to determine the use of this medium in identifying *V. parahaemolyticus* or *V. cholerae* and *V. vulnificus* isolates. Of these, 18 were mis-identified on CHROMagar: 12 isolates (4 × *V. calviensis*, 2 × *V. coralliilyticus*, 1 × *V. mytili*, 5 × *V. proteolyticus*) incorrectly produced mauve colonies indicative of *V. parahaemolyticus*; five isolates (2 × *V. calviensis*, 2 × *V. coralliilyticus*, 1 × *V. proteolyticus*) incorrectly produced aqua colonies indicative of either *V. cholerae* or *V. vulnificus;* 1 × *V. vulnificus* produced white colonies. All other isolates produced typical colonies (Table 17).

Table 17: Results from culturing 106 *Vibrio* isolates from the *Vibrio* strain library on *Vibrio* CHROMagar. Bracketed figures denote percentage of correct identifications.

Micro-organism	Mauve	Aqua	White
V. parahaemolyticus	29 (100%)	0	0
V. vulnificus	0	6 (86%)	1 (14%)
Other *Vibrio* sp.	12 (17%)	5 (7%)	53 (76%)

Traditionally, random growth positive tubes are streaked onto TCBS agar to check for colonies typical of the target organism. However, such methods require additional PCR or biochemical identification due to the semi-selective nature of TCBS agar that can take a significant amount of time. Based on these results CHROMagar has great potential as an alternative to TCBS agar for periodic assessment of MPN tubes that does not require the additional biochemical evaluation (except that required to distinguish *V. cholerae* from *V. vulnificus* in which case, growth on TCBS agar will be sufficient with *V. cholerae* producing yellow colonies due to producing acid from sucrose compared to small lime green colonies of *V .vulnificus*). In particular, no false negatives were produced from any of the *V. parahaemolyticus* isolates, although some 'over-scoring' may be possible from other

species (17%) that also produced mauve colonies typical of this organism. From a risk perspective, it is better to over-estimate the presence of these pathogens in a food sample than to under-estimate.

3.3.3 Accuracy of Real-Time Methods

Results are presented in Appendix 3. However, in summary all isolates (5 × *V. cholerae* (3 × *ctx*$^+$), 36 × *V. parahaemolyticus* (10 × *tdh*$^+$, 7 × *trh*$^+$), 3 × *V. vulnificus*, 58 × other *Vibrio* species and 8 × non-*Vibrio* species) were correctly identified by each of the Real-Time PCR methods. Given no false-positive or false-negative results were observed, the assessed Real-Time PCR methods for the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are highly accurate. Such an outcome will provide a high level of confidence in future results from prawn samples where a wide range of *Vibrio* species may be present.

3.3.4 Assessment of Real-Time PCR to Detect Pathogenic *Vibrio* Species in Mixed Culture

V. cholerae (*ctx*), *V. parahaemolyticus* (*tlh*) and *V. vulnificus* (*vvh*) was detected in all mixed cultures by Real-Time PCR. No false positives were detected in the negative control DNA lysates.

3.3.5 Assessment of Inoculated Prawns by Real-Time PCR

3.3.5.1 V. parahaemolyticus

Growth of the inoculated *V. parahaemolyticus* culture on TCBS suggested prawn inoculation levels of 8.4 cfu/g, 84 cfu/g 840 cfu/g and 8,400 cfu/g. Overall, the MPN-Real-Time PCR method provided highly linear results (r^2=0.92) (Figure 3) with the variance in the difference between the inoculum and the MPN results within the range expected (0.145).

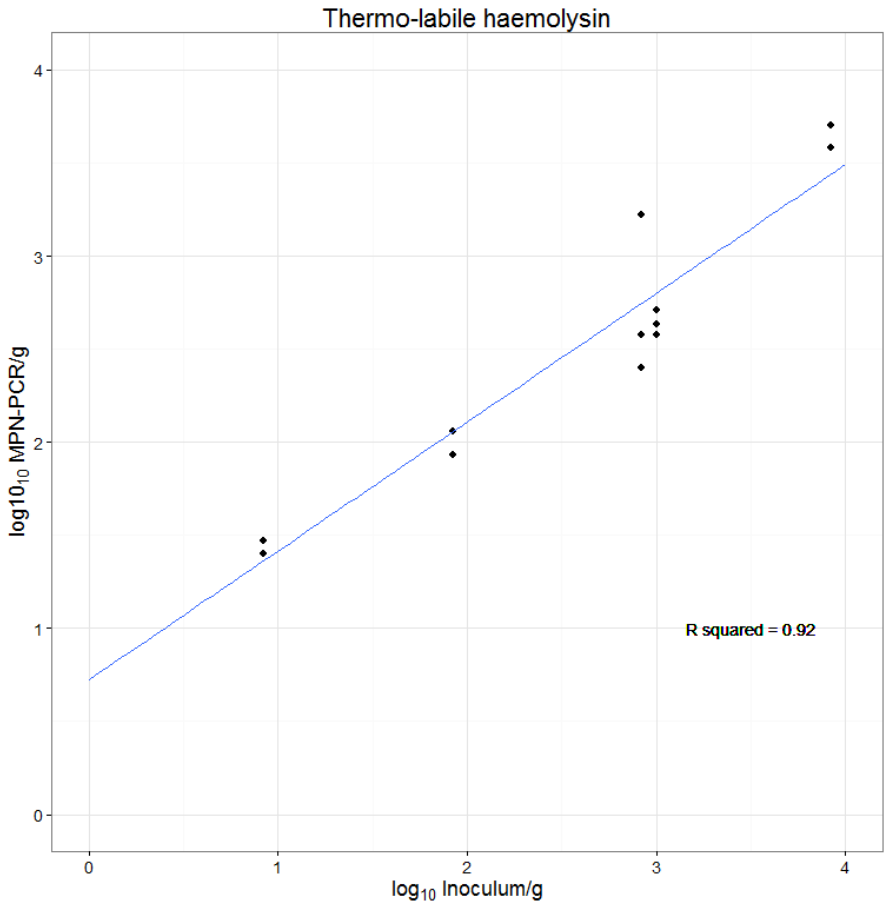

Figure 3: Linear relationship of the Log_{10} *V. parahaemolyticus* counts per gram (as determined by MPN-Real-Time PCR of the *tlh* gene) from inoculated prawn samples compared with the Log_{10} *V. parahaemolyticus* of the inoculum as determined by growth on TCBS agar.

3.3.5.2 V. vulnificus

Growth of the inoculated *V. vulnificus* culture on TCBS suggested prawn inoculation levels of 0.3 cfu/g, 3 cfu/g 28 cfu/g and 280 cfu/g. Overall, the MPN-Real-Time PCR method provided highly linear results (r^2=0.91) (Figure 4) with the variance in the difference between the inoculum and the MPN results within the range expected (0.082).

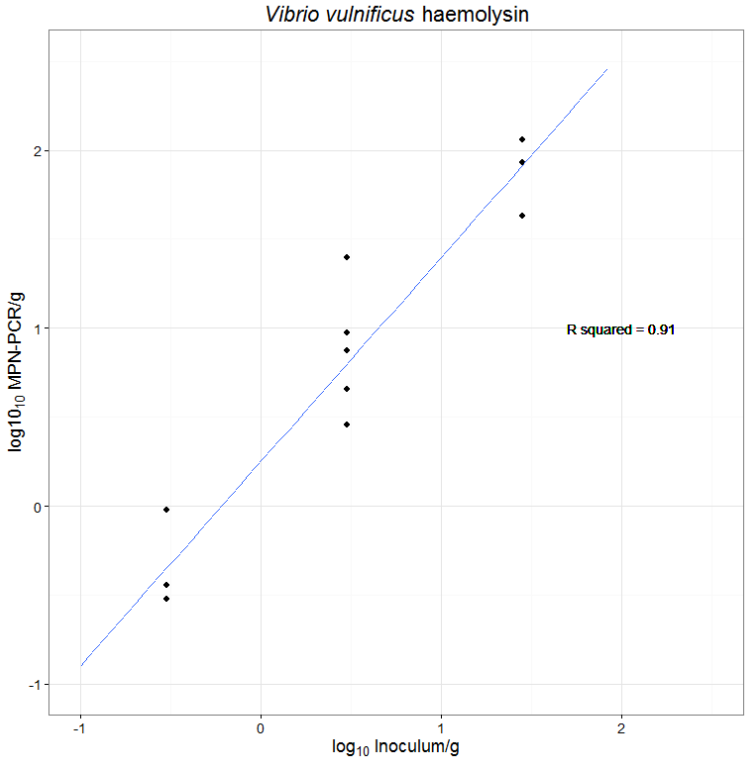

Figure 2: Linear relationship of the Log_{10} *V. vulnificus* counts per gram (as determined by MPN-Real-Time PCR of the *vvh* gene) from inoculated prawn samples compared with the Log_{10} *V. parahaemolyticus* of the inoculum as determined by growth on TCBS agar.

These results demonstrate that the MPN-Real-Time PCR methods for detecting pathogenic vibrios in prawns are suitable for use in assessing Australian prawn products with no inhibition of amplification seen in any of the samples. A demonstrated limit of detection of 0.3 MPN/g of prawn tissue for *V. vulnificus* is expected to be replicated for all target genes. This compares favourably to methods that require growth on TCBS (LoD = 100 cfu/g) or direct detection by Real-Time PCR or conventional PCR (>100 cfu/g). Such a method could also be made more sensitive by enriching 25 g of prawn tissue in 225 mL of APW as the first dilution, providing a sensitivity of 1 cfu/25 g, which equates to the proscribed limit for various seafood markets including China (*V. parahaemolyticus* not detected in 25 g of aquatic products), Thailand (*V. cholerae* not detected in 25 g of chilled, cooked, ready to eat shrimp or prawn) and the USA (*V. cholerae* not detected in 25 g of fishery products requiring minimal cooking by the consumer).

3.3.6 Australian Prawn Survey

All prawn samples tested had <0.3 MPN/g of *V. cholerae* and *V. vulnificus* (Table 18). All but one sample found to be below the level of detection (<0.3 MPN/g) for *V. parahaemolyticus*. The one positive sample (ACPF-003), a Western King Prawn sample from South Australia, had 0.3 MPN/g. The sample did not contain the *V. parahaemolyticus* virulence marker *tdh* or *trh*.

Table 18: Results obtained from 29 natural prawn samples tested for *V. cholerae* (*ctx*), *V. parahaemolyticus* (*tlh*) and *V. vulnificus* (*vvh*) by MPN-Real-Time PCR.

Sample No.	Species	Location	*ctx*	*tlh*	*vvh*
ACPF - 003	Western King	SA - Spencer Gulf	<0.3	0.3	<0.3
ACPF - 004	Western King	SA - Spencer Gulf	<0.3	<0.3	<0.3
ACPF - 006	Western King	SA - Spencer Gulf	<0.3	<0.3	<0.3
ACPF - 008	Western King	SA - Spencer Gulf	<0.3	<0.3	<0.3
ACPF - 011	Western King	SA - Spencer Gulf	<0.3	<0.3	<0.3
ACPF - 012	Western King	SA - Spencer Gulf	<0.3	<0.3	<0.3
ACPF - 015	Western King	SA - Spencer Gulf	<0.3	<0.3	<0.3
ACPF - 017	Blue endeavour	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 019	Brown tiger	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 020	Grooved tiger	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 024	Brown tiger	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 026	Blue endeavour	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 029	Brown tiger	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 033	Banana	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 034	Brown tiger	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 037	Blue leg king	QLD - Mackay	<0.3	<0.3	<0.3
ACPF - 116	Green tiger	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 120	Banana	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 127	Brown tiger	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF - 128	Green tiger	NPF- Gulf of Carpentaria	<0.3	<0.3	<0.3
ACPF – 053	Eastern King (cooked)	SE QLD	<0.3	<0.3	<0.3
ACPF – 055	Eastern King (cooked)	SE QLD	<0.3	<0.3	<0.3
ACPF – 056	Eastern King (cooked)	SE QLD	<0.3	<0.3	<0.3
ACPF – 057	Eastern King (cooked)	SE QLD	<0.3	<0.3	<0.3
ACPF – 059	Eastern King (cooked)	SE QLD	<0.3	<0.3	<0.3
ACPF – 086	Tiger	Shark Bay WA	<0.3	<0.3	<0.3
ACPF – 087	Tiger	Shark Bay WA	<0.3	<0.3	<0.3
ACPF – 089	Coral	Shark Bay WA	<0.3	<0.3	<0.3
ACPF – 096	King	Shark Bay WA	<0.3	<0.3	<0.3

These results suggest that pathogenic *Vibrio* species are of low risk in Australian wild caught prawns. This is in agreement with the risk ranking conducted as part of Seafood CRC Project

2009/787. However, given the small nature of the survey, a larger more comprehensive survey of Australian prawns is recommended.

3.4 Summary and Conclusion

Real-Time PCR results from pure cultures, mixed cultures and assessment of inoculated prawns provided consistent accurate results; regardless of which gene target was used. Given this, full confidence can be given to results obtained from natural prawn samples. The results from the Australian wild caught prawn survey suggest that pathogenic *Vibrio* species pose a low risk to consumers, however a larger, more comprehensive survey is recommended.

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References

Alsina, M. and R. Blanch (1994a). "A Set of Keys for Biochemical Identification of Environmental *Vibrio* Species." Journal of Applied Bacteriology **76**: 79-85.

Alsina, M. and R. Blanch (1994b). "Improvement and Update of a Set of Keys for biochemical Identification of *Vibrio* Species." Journal of Applied Bacteriology **77**: 719-722.

Anonymous (2004). "Bacteriological Analytical Manual." Retrieved December 16, 2011.

Anonymous (2010). Guidelines on the Application of General Principles of Food Hygiene to the Control of Pathogenic *Vibrio* Species in seafood, Food and Agriculture Organisation/World Health Organisation.

Arias, C., et al. (1999). "Low Incidence of *Vibrio vulnificus* Among *Vibrio* Isolates from Sea Water and Shellfish of the Western Mediterranean Coast." Journal of Applied Microbiology **86**: 125-134.

Austin, B., et al. (1995). "Identification and Typing of *Vibrio anguillarum*: A Comparison of Different Methods." Systematic and Applied Microbiology **18**(2): 285-302.

Baker-Austin, C., et al. (2010). "Rapid *in situ* Detection of Virulent *Vibrio vulnificus* Strains in Raw Oyster Matrices Using Real-Time PCR." Environmental Microbiology Reports **2**(1): 76-80.

Baker-Austin, C., et al. (2012). "*pilF* Polymorphism-Based Real-Time PCR to Distinguish *Vibrio vulnificus* Strains of Human Health Relevance." food Microbiology **30**: 17-23.

Bauer, A. and L. Rorvik (2007). "A Novel Multiplex PCR for the Identification of *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*." Letters in Applied Microbiology **45**: 371-375.

Bej, A., et al. (1999). "Detection of Total and Hemolysin-Producing *Vibrio parahaemolyticus* in Shellfish Using Multiplex PCR Amplification of *tl*, *tdh* and *trh*." Journal of Microbiological Methods **36**: 215-225.

Beuchat, L. (1977). "Suitability of Some Enrichment Broths and Diluents for Enumerating Cold and Heat Stressed *Vibrio parahaemolyticus*." Canadian Journal of Microbiology **23**: 630-633.

Biosca, E., et al. (1993). "Presence of a Capsule in *Vibrio vulnificus* Biotype 2 and its Relationship to Virulence for Eels." Infection and Immunity **61**: 1611-1618.

Blackstone, G., et al. (2007). "Use of a Real Time PCR Assay for Detection of th *ctx*A Gene of *Vibrio cholerae* in an Environmental Survey of Mobile Bay." Journal of Microbiological Methods **68**: 254-259.

Blackstone, G., et al. (2003). "Detection of Pathogenic *Vibrio parahaemolyticus* in Oyster Enrichments by Real Time PCR." Journal of Microbiological Methods **53**: 149-155.

Brasher, C., et al. (1998). "Detection of Microbial Pathogens in Shellfish with Multiplex PCR." Current Microbiology **37**: 101-107.

Brauns, L., et al. (1991). "Use of the Polymerae Chain Reaction in Detection of Culturable and Nonculturable *Vibrio vulnificus* Cells." Applied and Environmental Microbiology **57**(9): 2651-2655.

Brayton, P., et al. (1983). "New Selective Plating Medium for Isolation of *Vibrio vulnificus* Biogroup 1." Journal of Clinical Microbiology **17**(6): 1039-1044.

Broberg, C., et al. (2011). "*Vibrio parahaemolyticus* Cell Biology and Pathogenicity Determinants." Microbes and Infection **13**: 992-1001.

Caburlotto, G., et al. (2010). "Effect on Human Cells of Environmental *Vibrio parahaemolyticus* Strains Carrying Type III Secretion System 2." Infection and Immunity **78**(7): 3280-3287.

Cai, T., et al. (2006). "Application of Real-Time PCR for Quantitative Detection of *Vibrio parahaemolyticus* from Seafood in Eastern China." FEMS Immunology and Medical Microbiology **46**(2): 180-186.

Campbell, M. and A. Wright (2003). "Real-Time PCR Analysis of *Vibrio vulnificus* from Oysters." Applied and Environmental Microbiology **69**(12): 7137-7144.

Cerdá-Cuéllar, M., et al. (2000). "A selective Medium and a Specific Probe for Detection of *Vibrio vulnificus*." Applied and Environmental Microbiology **66**(2): 855-859.

Cho, Y., et al. (2010). "Genomic Evolution of *Vibrio cholerae*." Current Opinion in Microbiology **13**: 646-651.

Cowan, S. (1974). Cowan and Steel's Manual for the Identificaiton of Medical bacteria. Cambridge, Cambridge University Press.

Croci, L., et al. (2007). "Comparison of Different Biochemical and Molecular Methods for the Identification of *Vibrio parahaemolyticus*." Journal of Applied Microbiology **102**: 229-237.

Datta, S., et al. (2008). "Immunomagnetic Separation and Coagglutination of *Vibrio parahaemolyticus* with Anti-Flagellar Protein Monoclonal Antibody." Clinical and Vaccine Immunology **15**(10): 1541-1546.

DePaola, A., et al. (1997). "Evaluation of an Alkaline Phosphotase-Labelled DNA Probe for Enumeration of *Vibrio vulnificus* in Gulf Coast Oysters." Journal of Microbiological Methods **29**: 115-120.

DeRita, V. (2000). "Genomics Happens." Science **289**(5484): 1488-1489.

Desmarchelier, P. (2003). Pathogenic Vibrios. Foodborne Microorganisms of Public Health Significance. A. Hocking. Marrickville, NSW, 2204, Southwood Press Pty Ltd**:** 333-358.

Espineira, M., et al. (2010). "Validation of a Method for the Detection of Five Species, Serogroups, Biotypes and Virulence Factors of *Vibrio* by Multiplex PCR in Fish and Seafood." food Microbiology **27**(1): 122-131.

FAO/WHO (2011). Risk Assessment of *Vibrio parahaemolyticus* in Seafood. Rome, FAO/WHO.

Farmer, J. and F. Hickman-Brenner (1992). The Genera *Vibrio* and *Photobacterium*. The Prokaryotes. A. Balows, H. Trupper, M. Dworkin, W. Harder and K. Schleifer. New York, Springer-Verlag. **III:** 2952-3011.

Fedio, W., et al. (2007). "Rapid Detection of the *Vibrio cholerae ctx* Gene in Food Enrichments Using Real-Time Polymerase Chain Reaction." Journal of AOAC International **90**(5): 1278-1283.

Fields, P., et al. (1992). "Use of Polymerase Chain Reaction for Detection of Toxigenic *Vibrio cholerae* O1 Strains from the Latin American Cholera Epidemic." Journal of Clinical Microbiology **30**(8): 2118-2121.

Garrity, G., et al. (2004). Bergey's Manual of Systematic Bacteriology. New York, Springer-Verlag.

Ghosh, C., et al. (1997). "A Search for Cholera Toxin (CT), Toxin Coregulated Pilus (TCP) and Other Virulence Factors in Non-O1/Non-139 *Vibrio cholerae*." Microbial Pathogenesis **22**: 199-208.

Gugliandolo, C., et al. (2010). "Conventional and Molecular Methods to Detect Bacterial Pathogens in Mussels." Letters in Applied Microbiology **52**(1): 15-21.

Han, F. and B. Ge (2008). "Evaluation of a Loop-Mediated Isothermal Amplification Assay for Detecting *Vibrio vulnificus* in Raw Oysters." Foodborne Pathogens and Disease **5**: 311-320.

Han, F., et al. (2011). "Detecting Potentially Virulent *Vibrio vulnificus* Strains in Raw Oysters by Quantitative Loop-Mediated Isothermal Amplification." Applied and Environmental Microbiology **77**(8): 2589-2595.

Hara-Kudo, Y., et al. (2001). "Improved Method for Detection of *Vibrio parahaemolyticus* in Seafood." Applied and Environmental Microbiology **67**(12): 5819-5823.

Herrington, D., et al. (1988). "Toxin, Toxin-Coregulated Pili, and the *tox*R Regulon are Essential for *Vibrio cholerae* Pathogenesis in Humans." Journal of Experimental Medicine **168**: 1487-1492.

Hervio-Heath, D., et al. (2002). "Occurrence of Pathogenic Vibrios in Coastal Areas of France." Journal of Applied Microbiology **92**(6): 1123-1135.

Hill, W., et al. (1991). "Polymerase Chain Reaction Identification of *Vibrio vulnficus* in Artificially Contaminated Oysters." Applied and Environmental Microbiology **57**(3): 707-711.

Høi, l., et al. (1998). "Improved Isolation of *Vibrio vulnificus* from Seawater and sediment with Cellobiose-Colistin Agar." Applied and Environmental Microbiology **64**(5): 1721-1724.

Honda, T., et al. (1980). "Immunological Methods for Detection of kanagawa Phenomenon of *Vibrio parahaemolyticus*." Journal of Clinical Microbiology **11**(6): 600-603.

Hsu, W., et al. (1998). "Enhanced Broth Media for Selective Growth of *Vibrio vulnificus*." Applied and Environmental Microbiology **64**(7): 2701-2704.

Hwang, B., et al. (2010). "Polymerase Chain Reaction-Based Detection of Total and Specific *Vibrio* Species." Applied Biochemistry and Biotechnology **162**: 1187-1194.

ISO (1990). "ISO 8914:1990 Microbiology - General Guidance for the Detection of *Vibrio parahaemolyticus*." International Organisation for Standardisation: Geneva.

Izumiya, H., et al. (2011). "Multiplex PCR Assay for Identification of Three Major Pathogenic *Vibrio* spp., *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus*." Molecular and Cellular Probes **25**: 174-176.

Jadeja, R., et al. (2010). "Immunomagnetic Separation of *Vibrio vulnificus* with Antiflagellar Monoclonal Antibody." Journal of Food Protection **73**(7): 1288-1293.

Jiang, K., et al. (2012). "Development of a Loop-Mediated Isothermal Amplification (LAMP) for the Detection of F5 Fimbriae Gene in Enterotoxigenic *E. coli*." Current Microbiology **65**: 633-638.

Jones, J., et al. (2009). "Evaluation of DNA Colony Hybridisation and Real-Time PCR for Detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in Postharvest-Processed Oysters." Journal of Food Protection **72**(10): 2106-2109.

Jones, J., et al. (2012). "Biochemical, Serological, and Virulence Characterization of Clinical and Oyster *Vibrio parahaemolyticus* Isolates." Journal of Clinical Microbiology **50**(7): 2343-2352.

Joseph, S., et al. (1982). "*Virbio parahaemolyticus* and Related Halophilic Vibrios." Critical Reviews in Microbiology **10**: 77-124.

Kim, D., et al. (2008). "Application of the *rpoS* Gene for Species-Specific Detection of *Vibrio vulnificus* by Real-Time PCR." Journal of Microbiology and Biotechnology **18**(11): 1841-1847.

Kim, M. and H. Bang (2008). "Detection of Marine Pathegenic Bacterial *Vibrio* Species by Multiplex Polymerase Chain Reaction (PCR)." Journal of Environmental Biology **29**(4): 543-546.

Kim, M. and H. Jeong (2001). "Development of 16S rRNA Targeted PCR Methods for the Detection and Differentiation of *Vibrio vulnificus* in Marine Environments." Aquaculture **193**: 199-211.

Kim, Y., et al. (1999). "Identification of *Vibrio parahaemolyticus* Strains at the Species Level by PCR Targeted to the *toxR* Gene." Journal of Clinical Microbiology **37**(4): 1173-1177.

Kitaura, T., et al. (1983). "Halo Production by sulfatase Activity of *V. vulnificus* and *V. cholerae* O1 on a New Selective Sodium Dodecyl Sulfate-Containing Agar Medium: A Screening Marker in Environmental Surveillance." FEMS Microbiology Letters **17**(205-9).

Koch, W., et al. (1993). Detection of Enterotoxigenic *Vibrio cholerae* in Foods by the Polymerase Chain Reaction. FDA Bacteriological Analytical Manual. Gaithersburg, AOAC International.

Kong, R., et al. (2002). "Rapid Detection of Six Types of Bacterial Pathogens in Marine Waters by Multiplex PCR." Water Research **36**(2802-12).

Kourany, M. (1983). "Medium for Isolation and Differentiation of *Vibrio parahaemolyticus* and *Vibrio alginolyticus*." Applied and Environmental Microbiology **45**(1): 310-312.

Lalitha, P., et al. (2008). "Analysis of *lol*B Gene and Its Use in the Development of a PCR Assay for the Detection of *Vibrio cholerae*." Journal of Microbiological Methods **75**(1): 142-144.

Lee, C., et al. (1995). "Sequence of a Clones pR72H Fragment and Its Use for Detection of *Vibrio parahaemoltyicus* in Shellfish with the PCR." Applied and Environmental Microbiology **61**(4): 1311-1317.

Lee, J., et al. (2004). "Role of Flagellum and Motility in Pathogensis of *Vibrio vulnificus*." Infection

Lee, S., et al. (2000). "Selection for *In Vivo* Regulators of Bacterial Virulence." Proceedings of the National Academy of Science USA **98**: 6889-6894.

Lee, S., et al. (1998). "Direct Identification of *Vibrio vulnificus* in Clinical Specimens by Nested PCR." Journal of Clinical Microbiology **36**(10): 2887-2892.

Levin, R. (2006). "*Vibrio vulnificus*, a Notably Lethal Human Pathogen Derived From Seafood: A Review of its Pathogenicity, Subspecies Characterization and Molecular Methods of Detection." Food Biotechnology **19**: 69-94.

Lin, Z., et al. (1993). "*Vibrio parahaemolyticus* has a Homolog of the *Vibrio cholerae* toxRS Operon that Mediates Environmentally Induced Regulation of the Thermostable Direct Hemolysin Gene." Journal of Bacteriology **175**(12): 3844-3855.

Lobitz, B., et al. (2000). "Climate and Infectious Disease: Use of Remote Sensing for Detection of *Vibrio cholerae* by Indirect Measurement." Proceedings of the National Academy of Science USA **97**(4): 1438-1443.

Makino, K., et al. (2003). "Genome Sequence of *Vibrio parahaemolyticus*: a Pathogenic Mechanism Distinct From That of *V. cholerae*." Mechanisms of Disease **361**: 743-749.

Malorney, B., et al. (2003). "Standardization of Diagnostic PCR for the Detection of Foodborne Pathogens." International Journal of Food Microbiology **83**: 39-41.

Massad, G. and J. Oliver (1987). "New Selevtive and Differencitla Meduim for *Vibrio cholerae* and *Vibrio vulnificus*." Applied and Environmental Microbiology **53**(9): 2262-2264.

Maxwell, E., et al. (1991). "A Case of *Vibrio vulnificus* Septicaemia Acquired in Victoria." The Medical Journal of Australia **154**: 214-215.

McCarthy, S., et al. (1999). "Evaluation of Alkaline Phosphotase and Digoxigenin-Labelled Probes for Detection of the Thermolabile Hemolysin (*tlh*) Gene of *Vibrio parahaemolyticus*." Letters in Applied Microbiology **28**(66-70).

McCarthy, S., et al. (2000). "Evaluation of Nonisotopic DNA Hybridisation Methods for Detection of the tdh Gene of *Vibrio parahaemolyticus*." Journal of Food Protection **63**: 1660-1664.

McCormack, W., et al. (1974). "Evaluation of Thiosulfate-Citrate-Bile Salts-Sucrose Agar, a Selective Medium for the Isolation of *Vibrio cholerae* and Other Pathogenic Vibrios." The Journal of Infectious Diseases **129**(5): 497-500.

Mendes, C., et al. (2008). "Development of a Multiplex Single-Tube Nested PCR (MSTNPCR) Assay for *Vibrio cholerae* O1 Detection." Journal of Microbiological Methods **72**: 191-196.

Messelhausser, L., et al. (2010). "Detection and Differentiation of *Vibrio* spp in Seafood and Fish Samples with Cultural and Molecular Methods." International Journal of Food Microbiology **42**(360-4).

Miyamoto, Y., et al. (1969). "*In Vitro* Hemolytic Characteristic of *Vibrio parahaemolyticus*: Its Close Correlation with Human Pathogenicity." Journal of Bacteriology **100**(2): 1147-1149.

Monsur, K. (1961). "A Highly Selective Gelatin-Taurocholate-Ellurite Medium For the Isolation of Cholera Vibrios." Transactions of the Royal Society of Tropical Medicine and Hygiene **55**: 440-442.

Monsur, K. (1963). "Bacteriological Diagnosis of Cholera Under Field Conditions." Bulletin of the World Health Organisation **28**: 387-389.

Moreno, M. and M. Landgraf (1998). "Virulene Factors and Pathoginicity of *Vibrio vulnificus* Strains Isolaed from Seafood." Journal of Applied Microbiology **84**: 747-751.

Morrison, S., et al. (2012). "Pyrosequencing-Based Comparative Genome Analysis of *Vibrio vulnificus* Environmental Isolates." PLoS One **7**(5): ePub.

Murray, R., et al. (1984). Bergey's Manual of Systematic Bacteriology. Baltimore, Williams and Wilkins.

Nair, G. B., et al. (2007). "Global Dissemination of *Vibrio parahaemolyticus* Serotype 03:K6 and its Serovariants." Clinical Microbiology Reviews **20**(1): 39-48.

Nandi, B., et al. (2000). "Rapid Method for Species-Specific identification of *Vibrio cholerae* Using Primers Targeted to the Gene fo Outer Membrane Protein OmpW." Journal of Clinical Microbiology **38**(11): 4145-4151.

Nemoto, J., et al. (2011). "Development and Evaluation of a Loop-Mediated Isothermal Aplification Assay for Rapid and Sensitive Detection of *Vibrio parahaemolyticus*." Journal of Food Protection **74**(9): 1462-1467.

Neogi, S., et al. (2010). "A Highly Sensitive and Specific Multiplex PCR Assay for Simultaneous Detection of *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus*." Letters in Applied Microbiology **51**(3): 293-300.

Nhung, P., et al. (2007). "Rapid and Specific Identification of 5 Human Pathogenic *Vibrio* Species by Multiplex Polymerase Chain Reaction Targeted to *dnaJ* Gene." Diagnostic Microbiology and Infectious Disease **59**(3): 271-275.

Noguerola, I. and R. Blanch (2008). "Identification of *Vibrio* spp. With a set of Dichotomous Keys." Journal of Applied Microbiology **105**: 175-185.

Nordstrom, J., et al. (2007). "Development of a Multiplex Real-Time PCR Assay with an Internal Amplification Control for the Detection of Total and Pathogenic *Vibrio parahaemolyticus* Bacteria in Oysters." Applied and Environmental Microbiology **73**(18): 5840-5847.

Noriea III, N., et al. (2010). "Distribution of Type III Secretion Systems in *Vibrio parahaemolyticus* from the Northern Gulf of Mexico." Journal of Applied Microbiology **109**: 953-962.

O'Hara, C., et al. (2003). "Accuracy of Six Commercially Available Systems for Identification of Members of the Family *Vibrionaceae*." Journal of Clinical Microbiology **41**(12): 5654-5659.

Oberbeckmann, S., et al. (2010). "A Polyphasic Approach for the Differentiation of Environmental *Vibrio* Isolates from Temperate Waters." FEMS Microbiology Ecology **75**: 145-162.

Okura, J., et al. (1997). "Emergence of a Unique O3:K6 Cone of *Vibrio parahaemolyticus* in Calcutta, India, and Isolation of Stains from the Same Clonal Group from Southeast Asian Travelers Arriving in Japan." Journal of Clinical Microbiology **35**(12): 3150-3155.

Ottaviani, D., et al. (2009). "Prevalence and Virulence Properties of non-O1 non-O139 *Vibrio cholerae* Strains from Seafood and Clinical Samples Collected in Italy." International Journal of Food Microbiology **132**: 47-53.

Pace, J. L., et al. (1997). "Effect of Bile of *Vibrio parahaemolyticus*." Applied and Environmental Microbiology **63**(6): 2372-2377.

Panicker, G., et al. (2004). "Detection of Pathogenic *Vibrio* spp. in shellfish by Using Multiplex PCR and DNA Microarrays." Applied and Environmental Microbiology **70**(12): 7436-7444.

Park, J., et al. (2011). "Complete Genome Sequence of *Vibrio vulnificus* MO6-24/O." Journal of Bacteriology **193**(8): 2062-2063.

Pascual, J., et al. (2010). "Multilocus Sequence Analysis of the Central Clade of the Genus *Vibrio* by Using the 16S rRNA, *recA*, *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* Genes." International Journal of Systematic and Evolutionary Microbiobiology **60**: 154-165.

Raghunath, P., et al. (2008). "Detection and Molecular Characterisation of *Vibrio parahaemolyticus* Isolated from Seafood Harvested Along the Southwest Coast of India." food Microbiology **25**: 824-830.

Rivera, I., et al. (2001). "Genotypes Associated with Virulence in Environmental Isolates of *Vibrio cholerae*." Applied and Environmental Microbiology **67**(6): 2421-2429.

Robert-Pillot, A., et al. (2010). "Total and Pathogenic *Vibrio parahaemolyticus* in Shrimp: Fast and Reliable Quantification by Real-Time PCR." International Journal of Food Microbiology **143**: 190-197.

Roberts, D. and M. Greenwood (2003). Practical Food Microbiology. Massachusetts, Blackwell Publishing Pty Ltd.

Roig, F., et al. (2010). "*pilF* Polymorphism-Based PCR to Distinguish *Vibrio vulnificus* Strains Potentially Dangerous to Public Health." Applied and Environmental Microbiology **76**(5): 1328-1333.

Roozbehani, A., et al. (2012). "A Rapid and Reliable Species-Specific Identification of Clinical and Environmental Isolates of *Vibrio cholerae* Using a Three-Test Procedure and *rec*A Polymerase Chain Reaction." Indian Journal of Medical Research **30**(1): 39-43.

Rosche, T., et al. (2005). "A Rapid and Simple PCR Analysis Indicates There Are Two Subgroups of *Vibrio vulnificus* Which Correlate with Clinical or Environmental Isolation." Microbiology and Immunology **49**: 381-389.

Said, B., et al. (1994). "The Use of Gene Probes, Immunoassays and Tissue Culture for the Detection of Toxin in *Vibrio cholerae* non-O1." Journal of Medical Microbiology **40**: 31-36.

Sakata, J., et al. (2011). "Production and Characterization of a Novel Monoclonal Antibody Against *Vibrio parahaemolyticus* F_0F_1 ATP Synthase's Delta Subunit and Its Application for Rapid Identification of the Pathogen." Journal of Microbiological Methods **In Press**.

Sakazaki, R. (1963). Manual of the Examination for Enteropathogenic Halophilic Bacteria. Tokyo, Japan, Ministry of Health.

Sakazaki, R., et al. (1968). "Studies on the Enteropathogenic, Facultatively Halophilic Bacteria, *Vibrio parahaemolyticus*. I. Morphological, Cultural and Biochemical Propoerties and its Taxonomic Position." Japanese Journal of Medical Science and Biology **16**: 161-188.

Schoolnik, G. and F. Yildiz (2000). "The Complete Genome Sequence of *Vibrio cholerae*: A Tale of Two Chromosomes and of Two Lifestyles." Genome Biology **1**(3): 1061.1061-1063.

Shangkuan, Y., et al. (1995). "Multiplex Polymerase Chain Reaction to Detect Toxigenic *Vibrio cholerae* and to Biotype *Vibrio cholerae* O1." Journal of Applied Bacteriology **79**: 364-373.

Shimada, T., et al. (1990). "A New Selective Differential Agar Medium for Isolation of *Vibrio cholerae* O1: PMT (polymyxyn-mannose-tellurtie) Agar." Japanese Journal of Medical Science and Biology **43**: 37-41.

Shirai, H., et al. (**1990**). "Molecular Epidemiologic Evidence for Association of Thermostable Direct Hemolysin (TDH) and TDH-Related Hemolysin of *Vibrio parahaemolyticus* with Gastroenteritis." Infection and Immunity **58**: 3568-3573.

Simpson, L., et al. (1987). "Correlation Between Virulence and Colony Morphology in *Vibrio vulnificus*." Infection and Immunity **55**: 269-272.

Srisuk, C., et al. (2010). "Rapid and Sensitive Detection of *Vibrio cholerae* by Loop-Mediated Isothermal Amplification Targeted to the Gene of Outer Membrane Protein ompW." Letters in Applied Microbiology **50**(1): 36-42.

Stanley, C. and V. Harwood (2010). "The USe of Genetic Typing Methods to Discriminate Among Strains of *Vibrio cholerae, V. parahaemolyticus* and *V. vulnificus*." Microbiological Methods **93**(5): 1553-1569.

Sumner, J. (2011). Food Safety Risks Associated with Prawns Consumed in Australia, Seafood CRC Project 2009/787.

Tada, J., et al. (1992). "Detection of the Thermostable Direct Hemolysin Gene (*tdh*) and the Thermostable Direct Hemolysin-Related Hemolysin Gene (*trh*) of *Vibrio parahaemolyticus* by Polymerase Chain Reaction." Molecular and Cellular Probes **6**: 477-487.

Tamplin, M., et al. (1991). "Enzyme Immunoassay for Identificaiton of *Vibrio vulnificus* in Seawater, Sediment and Oysters." Applied and Environmental Microbiology **57**: 1235-1240.

Taniguchi, H., et al. (1990). "Cloning and Characterisation of a Gene Encoding a New Thermostable Hemolysin from *Vibrio parahaemolyticus*." FEMS Microbiology Letters **67**: 339-346.

Tantillo, G., et al. (2004). "Updated Perspectives on Emerging Vibrios Assosiated with Human Infections." Letters in Applied Microbiology **39**(117-26).

Tebbs, R., et al. (2011). "Design and Validation of a Novel Multiplex Real-Time PCR Assay for *Vibrio* Pathogen Detection." Journal of Food Protection **74**(6): 939-948.

Teh, C., et al. (2010). "Simultaneous Differential Detection of Human Pathogenic and Nonpathogenic *Vibrio* Species Using a Multiplex PCR based on *gyr*B and *pnt*A Genes." Journal of Applied Microbiology **108**(6): 1940-1945.

Thompson, F., et al. (2004). "Biodiversity of Vibrios." Microbiology and Molecular Reviews **68**(3): 403-431.

Urakawa, H., et al. (1999). "16S rDNA Restriction Fragment Length Polymorphism Analysis of Psychrotrophic Vibrios from japanese Coastal Water." Canadian Journal of Microbiology **45**: 1001-1007.

Vora, G., et al. (2005). "Microarray-Based Detection of Genetic Heterogeneity, Antimicrobial Resistance and the Viable But Non-Culturable State in Human Pathogenic Vibrio spp." Proclomations of the National Academy of Science USA **102**(52): 19109-19114.

Vuddhakul, V., et al. (2006). "Distribution of Virulent and Pandemic Strains of *Vibrio parahaemolyticus* in Three Molluscan Shellfish Species (*Meretrix meretrix, Perna viridis,* and *Anadara granosa*) and Their Association with Foodborne Disease in Southern Thailand." Journal of Food Protection **69**(11): 2615-2620.

Waldor, M. and J. Mekalanos (1996). "Lysogenic Conversion by a Filamentous Phage Encoding Cholera Toxin." Science **272**(5270): 1910-1914.

Wang, D., et al. (2011). "Rapid Detection of *Listeria monocytogenes* in Raw Milk with Loop-Mediated Isothermal Amplification and Chemosensor." Journal of Food Science **76**(9): 611-615.

Wang, R., et al. (2011). "Detection and Identification of *Vibrio parahaemolyticus* by Multiplex PCR and DNA-DNA Hybridisation on a Microarray." Journal of Genetics and Genomics **38**: 129-135.

Wong, F. and P. Desmarchelier (1999). *Vibrio cholerae*. *Vibrio*, Academic Press**:** 2242-2248.

Wright, A., et al. (1992). "Development and Testing of a Nonradioactive DNA Oligonucleotide Probe That is Specific for *Vibrio cholerae* Cholera Toxin." Journal of Clinical Microbiology **30**(9): 2302-2306.

Wright, A., et al. (1993). "Rapid Identification of *Vibrio vulnificus* on Nonselective Media with and Alkaline Phosphatase-Labelled Oligonucleotide Probe." Applied and Environmental Microbiology **59**(2): 541-546.

Wright, A., et al. (2001). "Identification of a Group 1-Like Capsular Polysaccharide Operon for *Vibrio vulnificus*." Infection and Immunity **69**(6893-6901).

Yam, W., et al. (1999). "Abundance of Clinical Enteric Bacterial Pathogens in Coastal Waters and Shellfish." Water Research **34**: 51-56.

Yamazaki, W., et al. (2010). "Development of Loop-Mediated Isothermal Amplification Assay for Sensitive and Rapid Detection of the *tdh* and *trh* Genes of *Vibrio parahaemlyticus* and Related *Vibrio* Species." Applied and Environmental Microbiology **76**(3): 820-828.

Yoh, M., et al. (1993). "Development of an Enzyme-Labelled Oligonucleotide Probe for the Cholera Toxin Gene." Journal of Clinical Microbiology **31**(5): 1312-1314.

Yoshida, S., et al. (1985). "Relation of Capsular Materials and Colony Opacity to Virulence of *Vibrio vulnificus*." Infection and Immunity **47**: 446-451.

Yu, S., et al. (2010). "Species-Specific PCR Detection of the Food-Borne Pathogen *Vibrio parahaemolyticus* Using the *irgB* Gene Identified by Comparative Genomic Analysis." FEMS Microbiology Letters **307**(1): 65-71.

Zuppardo, A. and R. Siebeling (1998). "An Epimerase Gene Essential for Capsule Synthesis in *Vibrio vulnificus*." Infection and Immunity **66**(2601-6).

Appendix 1: Loop-Mediated Isothermal Amplification and Conventional PCR Reaction Mixes and Results

Table A10: Reaction mixes and results for LAMP detection of *tlh* has per Nemoto *et al*., 2010

	Oligonucleotide Primers				Anneal	
Tube No.	F3 µM	B3 µM	FIP µM	BIP µM	Temp	Result
1	0.4	0.4	1.6	1.6	63 deg	Positive
2	0.4	0.4	1.6	1.6	63 deg	Positive
3	0.4	0.4	1.1	1.1	63 deg	Positive
4	0.4	0.4	1.1	1.1	63 deg	Positive
5	0.4	0.4	2.1	2.1	63 deg	Negative
6	0.4	0.4	2.1	2.1	63 deg	Negative
7	0.4	0.4	1.6	1.6	67 deg	Positive
8	0.4	0.4	1.6	1.6	67 deg	Positive
9	0.4	0.4	1.1	1.1	67 deg	Negative
10	0.4	0.4	1.1	1.1	67 deg	Negative
11	0.4	0.4	2.1	2.1	67 deg	Negative
12	0.4	0.4	2.1	2.1	67 deg	Negative
13	0.4	0.4	1.6	1.6	65 deg	Positive
14	0.4	0.4	1.6	1.6	65 deg	Positive
15	0.4	0.4	1.1	1.1	65 deg	Positive
16	0.4	0.4	1.1	1.1	65 deg	Negative
17	0.4	0.4	2.1	2.1	65 deg	Negative
18	0.4	0.4	2.1	2.1	65 deg	Negative
19	0.09	0.09	1.6	1.6	63 deg	Negative
20	0.09	0.09	1.6	1.6	63 deg	Positive
21	0.09	0.09	1.1	1.1	63 deg	Negative
22	0.09	0.09	1.1	1.1	63 deg	Positive
23	0.09	0.09	2.1	2.1	63 deg	Negative
24	0.09	0.09	2.1	2.1	63deg	Negative
25	0.09	0.09	1.6	1.6	67 deg	Positive
26	0.09	0.09	1.6	1.6	67 deg	Positive
27	0.09	0.09	1.1	1.1	67 deg	Negative
28	0.09	0.09	1.1	1.1	67 deg	Positive
29	0.09	0.09	2.1	2.1	67 deg	Negative
30	0.09	0.09	2.1	2.1	67 deg	Negative
31	0.09	0.09	1.6	1.6	65 deg	Negative

	Oligonucleotide Primers				Anneal	
Tube No.	**F3 µM**	**B3 µM**	**FIP µM**	**BIP µM**	**Temp**	**Result**
32	0.09	0.09	1.6	1.6	65 deg	Negative
33	**0.09**	**0.09**	**1.1**	**1.1**	**65 deg**	Positive
34	**0.09**	**0.09**	**1.1**	**1.1**	**65 deg**	Negative
35	0.09	0.09	2.1	2.1	65 deg	Negative
36	0.09	0.09	2.1	2.1	65 deg	Negative
37	**0.9**	**0.9**	**1.6**	**1.6**	**63 deg**	Positive
38	**0.9**	**0.9**	**1.6**	**1.6**	**63 deg**	Negative
39	0.9	0.9	1.1	1.1	63 deg	Negative
40	0.9	0.9	1.1	1.1	63 deg	Negative
41	**0.9**	**0.9**	**2.1**	**2.1**	**63 deg**	Positive
42	**0.9**	**0.9**	**2.1**	**2.1**	**63 deg**	Negative
43	0.9	0.9	1.6	1.6	67 deg	Positive
44	0.9	0.9	1.6	1.6	67 deg	Negative
45	**0.9**	**0.9**	**1.1**	**1.1**	**67 deg**	Negative
46	**0.9**	**0.9**	**1.1**	**1.1**	**67 deg**	Negative
47	0.9	0.9	2.1	2.1	67 deg	Negative
48	0.9	0.9	2.1	2.1	67 deg	Negative
49	**0.9**	**0.9**	**1.6**	**1.6**	**65 deg**	Positive
50	**0.9**	**0.9**	**1.6**	**1.6**	**65 deg**	Positive
51	0.9	0.9	1.1	1.1	65 deg	Negative
52	0.9	0.9	1.1	1.1	65 deg	Positive
53	**0.9**	**0.9**	**2.1**	**2.1**	**65 deg**	Negative
54	**0.9**	**0.9**	**2.1**	**2.1**	**65 deg**	Positive

Table A11: Reaction mixes and results for LAMP detection of *tdh* has per Yamazaki *et al*., 2010

	Oligonucleotide Primers				Anneal	
Tube No.	F3 µM	B3 µM	FIP µM	BIP µM	Temp	Result
1	0.8	0.8	0.2	0.2	63 deg	Negative
2	0.8	0.8	0.2	0.2	63 deg	Positive
3	0.8	0.8	0.07	0.07	63 deg	Positive
4	0.8	0.8	0.07	0.07	63 deg	Negative
5	0.8	0.8	0.7	0.7	63 deg	Negative
6	0.8	0.8	0.7	0.7	63 deg	Negative
7	0.8	0.8	0.2	0.2	67 deg	Positive
8	0.8	0.8	0.2	0.2	67 deg	Positive
9	0.8	0.8	0.07	0.07	67 deg	Negative
10	0.8	0.8	0.07	0.07	67 deg	Negative
11	0.8	0.8	0.7	0.7	67 deg	Negative
12	0.8	0.8	0.7	0.7	67 deg	Negative
13	0.8	0.8	0.2	0.2	65 deg	Positive
14	0.8	0.8	0.2	0.2	65 deg	Negative
15	0.8	0.8	0.07	0.07	65 deg	Negative
16	0.8	0.8	0.07	0.07	65 deg	Positive
17	0.8	0.8	0.7	0.7	65 deg	Positive
18	0.8	0.8	0.7	0.7	65 deg	Positive
19	0.3	0.3	0.2	0.2	63 deg	Negative
20	0.3	0.3	0.2	0.2	63 deg	Negative
21	0.3	0.3	0.07	0.07	63 deg	Positive
22	0.3	0.3	0.07	0.07	63 deg	Positive
23	0.3	0.3	0.7	0.7	63 deg	Negative
24	0.3	0.3	0.7	0.7	63deg	Negative
25	0.3	0.3	0.2	0.2	67 deg	Negative
26	0.3	0.3	0.2	0.2	67 deg	Negative
27	0.3	0.3	0.07	0.07	67 deg	Negative
28	0.3	0.3	0.07	0.07	67 deg	Positive
29	0.3	0.3	0.7	0.7	67 deg	Negative
30	0.3	0.3	0.7	0.7	67 deg	Negative
31	0.3	0.3	0.2	0.2	65 deg	Negative
32	0.3	0.3	0.2	0.2	65 deg	Negative
33	0.3	0.3	0.07	0.07	65 deg	Positive
34	0.3	0.3	0.07	0.07	65 deg	Positive

	Oligonucleotide Primers				Anneal	
Tube No.	**F3 µM**	**B3 µM**	**FIP µM**	**BIP µM**	**Temp**	**Result**
35	0.3	0.3	0.7	0.7	65 deg	Negative
36	0.3	0.3	0.7	0.7	65 deg	Positive
37	**1.3**	**1.3**	**0.2**	**0.2**	**63 deg**	Positive
38	**1.3**	**1.3**	**0.2**	**0.2**	**63 deg**	Negative
39	1.3	1.3	0.07	0.07	63 deg	Positive
40	1.3	1.3	0.07	0.07	63 deg	Negative
41	**1.3**	**1.3**	**0.7**	**0.7**	**63 deg**	Positive
42	**1.3**	**1.3**	**0.7**	**0.7**	**63 deg**	Negative
43	1.3	1.3	0.2	0.2	67 deg	Positive
44	1.3	1.3	0.2	0.2	67 deg	Negative
45	**1.3**	**1.3**	**0.07**	**0.07**	**67 deg**	Negative
46	**1.3**	**1.3**	**0.07**	**0.07**	**67 deg**	Negative
47	1.3	1.3	0.7	0.7	67 deg	Negative
48	1.3	1.3	0.7	0.7	67 deg	Negative
49	**1.3**	**1.3**	**0.2**	**0.2**	**65 deg**	Positive
50	**1.3**	**1.3**	**0.2**	**0.2**	**65 deg**	Positive
51	1.3	1.3	0.07	0.07	65 deg	Positive
52	1.3	1.3	0.07	0.07	65 deg	Negative
53	**1.3**	**1.3**	**0.7**	**0.7**	**65 deg**	Negative
54	**1.3**	**1.3**	**0.7**	**0.7**	**65 deg**	Negative

Table A12: Reaction mixes and results for LAMP detection of *trh* has per Yamazaki *et al*., 2010

	Oligonucleotide Primers				Anneal	
Tube No.	F3 µM	B3 µM	FIP µM	BIP µM	Temp	Result
1	0.4	0.4	1.6	1.6	63 deg	Positive
2	0.4	0.4	1.6	1.6	63 deg	Positive
3	0.4	0.4	1.1	1.6	63 deg	Positive
4	0.4	0.4	1.1	1.6	63 deg	Negative
5	0.4	0.4	2.1	60	63 deg	Negative
6	0.4	0.4	2.1	60	63 deg	Negative
7	0.4	0.4	1.6	40	67 deg	Positive
8	0.4	0.4	1.6	40	67 deg	Positive
9	0.4	0.4	1.1	20	67 deg	Negative
10	0.4	0.4	1.1	20	67 deg	Negative
11	0.4	0.4	2.1	60	67 deg	Negative
12	0.4	0.4	2.1	60	67 deg	Negative
13	0.4	0.4	1.6	40	65 deg	Positive
14	0.4	0.4	1.6	40	65 deg	Positive
15	0.4	0.4	1.1	20	65 deg	Negative
16	0.4	0.4	1.1	20	65 deg	Positive
17	0.4	0.4	2.1	60	65 deg	Negative
18	0.4	0.4	2.1	60	65 deg	Negative
19	0.09	0.09	1.6	40	63 deg	Negative
20	0.09	0.09	1.6	40	63 deg	Negative
21	0.09	0.09	1.1	20	63 deg	Positive
22	0.09	0.09	1.1	20	63 deg	Positive
23	0.09	0.09	2.1	60	63 deg	Negative
24	0.09	0.09	2.1	60	63deg	Negative
25	0.09	0.09	1.6	40	67 deg	Positive
26	0.09	0.09	1.6	40	67 deg	Negative
27	0.09	0.09	1.1	20	67 deg	Negative
28	0.09	0.09	1.1	20	67 deg	Positive
29	0.09	0.09	2.1	60	67 deg	Negative
30	0.09	0.09	2.1	60	67 deg	Negative
31	0.09	0.09	1.6	40	65 deg	Negative
32	0.09	0.09	1.6	40	65 deg	Negative
33	0.09	0.09	1.1	20	65 deg	Positive
34	0.09	0.09	1.1	20	65 deg	Positive

	Oligonucleotide Primers				Anneal	
Tube No.	**F3 µM**	**B3 µM**	**FIP µM**	**BIP µM**	**Temp**	**Result**
35	0.09	0.09	2.1	60	65 deg	Negative
36	0.09	0.09	2.1	60	65 deg	Positive
37	**0.9**	**0.9**	**1.6**	**40**	**63 deg**	Positive
38	**0.9**	**0.9**	**1.6**	**40**	**63 deg**	Negative
39	0.9	0.9	1.1	20	63 deg	Positive
40	0.9	0.9	1.1	20	63 deg	Negative
41	**0.9**	**0.9**	**2.1**	**60**	**63 deg**	Positive
42	**0.9**	**0.9**	**2.1**	**60**	**63 deg**	Negative
43	0.9	0.9	1.6	40	67 deg	Positive
44	0.9	0.9	1.6	40	67 deg	Negative
45	**0.9**	**0.9**	**1.1**	**20**	**67 deg**	Negative
46	**0.9**	**0.9**	**1.1**	**20**	**67 deg**	Negative
47	0.9	0.9	2.1	60	67 deg	Negative
48	0.9	0.9	2.1	60	67 deg	Negative
49	**0.9**	**0.9**	**1.6**	**40**	**65 deg**	Positive
50	**0.9**	**0.9**	**1.6**	**40**	**65 deg**	Positive
51	0.9	0.9	1.1	20	65 deg	Positive
52	0.9	0.9	1.1	20	65 deg	Positive
53	**0.9**	**0.9**	**2.1**	**60**	**65 deg**	Positive
54	**0.9**	**0.9**	**2.1**	**60**	**65 deg**	Positive

Table A13: Reaction mixes and results for LAMP detection of *vvh* has per Han & Ge, 2008

	Oligonucleotide Primers				Anneal	
Tube No.	F3 µM	B3 µM	FIP µM	BIP µM	Temp	Result
1	0.2	0.2	1.6	1.6	63 deg	Negative
2	0.2	0.2	1.6	1.6	63 deg	Negative
3	0.2	0.2	1.1	1.1	63 deg	Negative
4	0.2	0.2	1.1	1.1	63 deg	Negative
5	0.2	0.2	2.1	2.1	63 deg	Negative
6	0.2	0.2	2.1	2.1	63 deg	Negative
7	0.2	0.2	1.6	1.6	61 deg	Negative
8	0.2	0.2	1.6	1.6	61 deg	Negative
9	0.2	0.2	1.1	1.1	61 deg	Negative
10	0.2	0.2	1.1	1.1	61 deg	Negative
11	0.2	0.2	2.1	2.1	61 deg	Negative
12	0.2	0.2	2.1	2.1	61 deg	Negative
13	0.2	0.2	1.6	1.6	65 deg	Negative
14	0.2	0.2	1.6	1.6	65 deg	Negative
15	0.2	0.2	1.1	1.1	65 deg	Negative
16	0.2	0.2	1.1	1.1	65 deg	Negative
17	0.2	0.2	2.1	2.1	65 deg	Negative
18	0.2	0.2	2.1	2.1	65 deg	Negative
19	0.07	0.07	1.6	1.6	63 deg	Negative
20	0.07	0.07	1.6	1.6	63 deg	Positive
21	0.07	0.07	1.1	1.1	63 deg	Negative
22	0.07	0.07	1.1	1.1	63 deg	Positive
23	0.07	0.07	2.1	2.1	63 deg	Negative
24	0.07	0.07	2.1	2.1	63deg	Negative
25	0.07	0.07	1.6	1.6	61 deg	Negative
26	0.07	0.07	1.6	1.6	61 deg	Negative
27	0.07	0.07	1.1	1.1	61 deg	Negative
28	0.07	0.07	1.1	1.1	61 deg	Negative
29	0.07	0.07	2.1	2.1	61 deg	Negative
30	0.07	0.07	2.1	2.1	61 deg	Negative
31	0.07	0.07	1.6	1.6	65 deg	Negative
32	0.07	0.07	1.6	1.6	65 deg	Negative
33	0.07	0.07	1.1	1.1	65 deg	Negative
34	0.07	0.07	1.1	1.1	65 deg	Negative

	Oligonucleotide Primers				Anneal	
Tube No.	**F3 µM**	**B3 µM**	**FIP µM**	**BIP µM**	**Temp**	**Result**
35	0.07	0.07	2.1	2.1	65 deg	Negative
36	0.07	0.07	2.1	2.1	65 deg	Negative
37	**0.7**	**0.7**	**1.6**	**1.6**	**63 deg**	Negative
38	**0.7**	**0.7**	**1.6**	**1.6**	**63 deg**	Negative
39	0.7	0.7	1.1	1.1	63 deg	Negative
40	0.7	0.7	1.1	1.1	63 deg	Negative
41	**0.7**	**0.7**	**2.1**	**2.1**	**63 deg**	Positive
42	**0.7**	**0.7**	**2.1**	**2.1**	**63 deg**	Negative
43	0.7	0.7	1.6	1.6	61 deg	Positive
44	0.7	0.7	1.6	1.6	61 deg	Negative
45	**0.7**	**0.7**	**1.1**	**1.1**	**61 deg**	Negative
46	**0.7**	**0.7**	**1.1**	**1.1**	**61 deg**	Negative
47	0.7	0.7	2.1	2.1	61 deg	Negative
48	0.7	0.7	2.1	2.1	61 deg	Negative
49	**0.7**	**0.7**	**1.6**	**1.6**	**65 deg**	Negative
50	**0.7**	**0.7**	**1.6**	**1.6**	**65 deg**	Negative
51	0.7	0.7	1.1	1.1	65 deg	Negative
52	0.7	0.7	1.1	1.1	65 deg	Negative
53	**0.7**	**0.7**	**2.1**	**2.1**	**65 deg**	Negative
54	**0.7**	**0.7**	**2.1**	**2.1**	**65 deg**	Positive

Table A14: Reaction mixes and results used to assess the robustness of conventional PCR to detect the *ctx* gene of virulent *V. cholerae* as per Koch *et al*., 1993.

	Oligonucleotide Primers								
Tube No.	Forward (µM)	Reverse (µM)	Buffer (µL)	$MgCl_2$	dNTPs (µL)	Taq (µL)	Water (µL)	Anneal Temp	Results
1	0.5	0.5	2.5	1.5 mM	0.5	0.5	17.25	55 deg	Good
2	0.5	0.5	2.5	1.5 mM	0.5	0.5	17.25	55 deg	Good
3	0.5	0.5	2.5	2.5 mM	0.5	0.5	16.75	55 deg	Good
4	0.5	0.5	2.5	2.5 mM	0.5	0.5	16.75	55 deg	Good
5	0.5	0.5	2.5	0.5 mM	0.5	0.5	15.5	55 deg	NR
6	0.5	0.5	2.5	0.5 mM	0.5	0.5	15.5	55 deg	Good
7	0.5	0.5	2.5	1.5 mM	0.5	0.5	17.25	57 deg	NR
8	0.5	0.5	2.5	1.5 mM	0.5	0.5	17.25	57 deg	2 Products
9	0.5	0.5	2.5	2.5 mM	0.5	0.5	16.75	57 deg	Weak
10	0.5	0.5	2.5	2.5 mM	0.5	0.5	16.75	57 deg	2 Products
11	0.5	0.5	2.5	0.5 mM	0.5	0.5	15.5	57 deg	NR
12	0.5	0.5	2.5	0.5 mM	0.5	0.5	15.5	57 deg	Good
13	0.5	0.5	2.5	1.5 mM	0.5	0.5	17.25	53 deg	Good
14	0.5	0.5	2.5	1.5 mM	0.5	0.5	17.25	53 deg	Good
15	0.5	0.5	2.5	2.5 mM	0.5	0.5	16.75	53 deg	Good
16	0.5	0.5	2.5	2.5 mM	0.5	0.5	16.75	53 deg	Many products
17	0.5	0.5	2.5	0.5 mM	0.5	0.5	15.5	53 deg	NR
18	0.5	0.5	2.5	0.5 mM	0.5	0.5	15.5	53 deg	NR
19	0.1	0.1	2.5	1.5 mM	0.5	0.5	19.25	55 deg	Good
20	0.1	0.1	2.5	1.5 mM	0.5	0.5	19.25	55 deg	Good

Tube No.	Oligonucleotide Primers: Forward (µM)	Oligonucleotide Primers: Reverse (µM)	Buffer (µL)	$MgCl_2$	dNTPs (µL)	Taq (µL)	Water (µL)	Anneal Temp	Results
21	0.1	0.1	2.5	2.5 mM	0.5	0.5	18.75	55 deg	Good
22	0.1	0.1	2.5	2.5 mM	0.5	0.5	18.75	55 deg	Good
23	0.1	0.1	2.5	0.5 mM	0.5	0.5	17.5	55 deg	NR
24	0.1	0.1	2.5	0.5 mM	0.5	0.5	17.5	55 deg	NR
25	0.1	0.1	2.5	1.5 mM	0.5	0.5	19.25	57 deg	Weak
26	0.1	0.1	2.5	1.5 mM	0.5	0.5	19.25	57 deg	Good
27	0.1	0.1	2.5	2.5 mM	0.5	0.5	18.75	57 deg	Good
28	0.1	0.1	2.5	2.5 mM	0.5	0.5	18.75	57 deg	Good
29	0.1	0.1	2.5	0.5 mM	0.5	0.5	17.5	57 deg	NR
30	0.1	0.1	2.5	0.5 mM	0.5	0.5	17.5	57 deg	NR
31	0.1	0.1	2.5	1.5 mM	0.5	0.5	19.25	53 deg	Good
32	0.1	0.1	2.5	1.5 mM	0.5	0.5	19.25	53 deg	Good
33	0.1	0.1	2.5	2.5 mM	0.5	0.5	18.75	53 deg	Good
34	0.1	0.1	2.5	2.5 mM	0.5	0.5	18.75	53 deg	Good
35	0.1	0.1	2.5	0.5 mM	0.5	0.5	17.5	53 deg	NR
36	0.1	0.1	2.5	0.5 mM	0.5	0.5	17.5	53 deg	NR
37	1	1	2.5	1.5 mM	0.5	0.5	14.75	55 deg	Good
38	1	1	2.5	1.5 mM	0.5	0.5	14.75	55 deg	Good
39	1	1	2.5	2.5 mM	0.5	0.5	14.25	55 deg	2 products
40	1	1	2.5	2.5 mM	0.5	0.5	14.25	55 deg	2 Products
41	1	1	2.5	0.5 mM	0.5	0.5	13	55 deg	NR

Tube No.	Oligonucleotide Primers		Buffer (µL)	$MgCl_2$	dNTPs (µL)	Taq (µL)	Water (µL)	Anneal Temp	Results
	Forward (µM)	Reverse (µM)							
42	1	1	2.5	0.5 mM	0.5	0.5	13	55 deg	NR
43	1	1	2.5	1.5 mM	0.5	0.5	14.75	57 deg	Good
44	1	1	2.5	1.5 mM	0.5	0.5	14.75	57 deg	Good
45	1	1	2.5	2.5 mM	0.5	0.5	14.25	57 deg	2 products
46	1	1	2.5	2.5 mM	0.5	0.5	14.25	57 deg	2 Products
47	1	1	2.5	0.5 mM	0.5	0.5	13	57 deg	NR
48	1	1	2.5	0.5 mM	0.5	0.5	13	57 deg	NR
49	1	1	2.5	1.5 mM	0.5	0.5	14.75	53 deg	NR
50	1	1	2.5	1.5 mM	0.5	0.5	14.75	53 deg	NR
51	1	1	2.5	2.5 mM	0.5	0.5	14.25	53 deg	2 products
52	1	1	2.5	2.5 mM	0.5	0.5	14.25	53 deg	NR
53	1	1	2.5	0.5 mM	0.5	0.5	13	53 deg	NR
54	1	1	2.5	0.5 mM	0.5	0.5	13	53 deg	NR

Table A1.6: Reaction mixes and results used to assess the robustness of conventional PCR to detect the *ctx* gene of virulent *V. cholerae* as per Bej *et al*., 1999

	Oligonucleotide Primers								
Tube No.	tl-F (µM)	tl-R (µM)	Buffer (µL)	$MgCl_2$	dNTPs	Taq (µL)	Water (µL)	Anneal Temp (°C)	Product
1	1	1	2.5	4 mM	0.5	0.5	16	58 deg	smear*
2	1	1	2.5	4 mM	0.5	0.5	16	58 deg	smear
3	1	1	2.5	3 mM	0.5	0.5	16.5	58 deg	smear
4	1	1	2.5	3 mM	0.5	0.5	16.5	58 deg	smear
5	1	1	2.5	2 mM	0.5	0.5	17	58 deg	good
6	1	1	2.5	2 mM	0.5	0.5	17	58 deg	good
7	1	1	2.5	4 mM	0.5	0.5	16	60 deg	low/byproduct*
8	1	1	2.5	4 mM	0.5	0.5	16	60 deg	low/byproduct
9	1	1	2.5	3 mM	0.5	0.5	16.5	60 deg	poor/byproduct
10	1	1	2.5	3 mM	0.5	0.5	16.5	60 deg	good
11	1	1	2.5	2 mM	0.5	0.5	17	60 deg	none/byproduct
12	1	1	2.5	2 mM	0.5	0.5	17	60 deg	low/byproduct
13	1	1	2.5	4 mM	0.5	0.5	16	56 deg	smear
14	1	1	2.5	4 mM	0.5	0.5	16	56 deg	smear
15	1	1	2.5	3 mM	0.5	0.5	16.5	56 deg	low/byproduct
16	1	1	2.5	3 mM	0.5	0.5	16.5	56 deg	smear/byproduct
17	1	1	2.5	2 mM	0.5	0.5	17	56 deg	poor/byproduct
18	1	1	2.5	2 mM	0.5	0.5	17	56 deg	good
19	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	58 deg	good

Tube No.	Oligonucleotide Primers tl-F (µM)	tl-R (µM)	Buffer (µL)	$MgCl_2$	dNTPs	Taq (µL)	Water (µL)	Anneal Temp (°C)	Product
20	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	58 deg	good
21	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	58 deg	good
22	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	58 deg	good
23	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	58 deg	low/byproduct
24	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	58 deg	good
25	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	60 deg	none/byproduct
26	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	60 deg	good
27	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	60 deg	good
28	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	60 deg	good
29	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	60 deg	good
30	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	60 deg	good/byproduct
31	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	56 deg	low/byproduct
32	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	56 deg	smear
33	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	56 deg	low/byproduct
34	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	56 deg	good/byproduct
35	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	56 deg	low
36	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	56 deg	smear
37	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	58 deg	good
38	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	58 deg	good
39	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	58 deg	good/byproduct
40	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	58 deg	none
41	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	58 deg	good

Tube No.	Oligonucleotide Primers tl-F (µM)	tl-R (µM)	Buffer (µL)	$MgCl_2$	dNTPs	Taq (µL)	Water (µL)	Anneal Temp (°C)	Product
42	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	58 deg	good
43	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	60 deg	good
44	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	60 deg	good
45	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	60 deg	good
46	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	60 deg	good/byproduct
47	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	60 deg	good
48	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	60 deg	good
49	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	56 deg	good
50	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	56 deg	good
51	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	56 deg	good
52	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	56 deg	good/byproduct
53	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	56 deg	low
54	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	56 deg	smear

* byproduct size ~75 bp

Table A1.7: Reaction mixes and results used to assess the robustness of conventional PCR to detect the *ctx* gene of virulent *V. cholerae* as per Bej *et al*., 1999

	Oligonucleotide Primers								
Tube No.	Forward (µM)	Reverse (µM)	Buffer (µL)	MgCl2	dNTPs (µL)	Taq (µL)	Water (µL)	Anneal Temp	Results
1	1	1	2.5	4 mM	0.5	0.5	16	63 deg	Smear
2	1	1	2.5	4 mM	0.5	0.5	16	63 deg	Smear
3	1	1	2.5	3 mM	0.5	0.5	16.5	63 deg	Smear
4	1	1	2.5	3 mM	0.5	0.5	16.5	63 deg	Smear
5	1	1	2.5	2 mM	0.5	0.5	17	63 deg	Smear
6	1	1	2.5	2 mM	0.5	0.5	17	63 deg	Smear
7	1	1	2.5	4 mM	0.5	0.5	16	65 deg	Good
8	1	1	2.5	4 mM	0.5	0.5	16	65 deg	Good
9	1	1	2.5	3 mM	0.5	0.5	16.5	65 deg	Good
10	1	1	2.5	3 mM	0.5	0.5	16.5	65 deg	NR
11	1	1	2.5	2 mM	0.5	0.5	17	65 deg	Good
12	1	1	2.5	2 mM	0.5	0.5	17	65 deg	NR
13	1	1	2.5	4 mM	0.5	0.5	16	67 deg	Smear
14	1	1	2.5	4 mM	0.5	0.5	16	67 deg	Smear
15	1	1	2.5	3 mM	0.5	0.5	16.5	67 deg	Smear
16	1	1	2.5	3 mM	0.5	0.5	16.5	67 deg	Smear
17	1	1	2.5	2 mM	0.5	0.5	17	67 deg	Smear
18	1	1	2.5	2 mM	0.5	0.5	17	67 deg	Smear

Tube No.	Oligonucleotide Primers Forward (µM)	Oligonucleotide Primers Reverse (µM)	Buffer (µL)	MgCl2	dNTPs (µL)	Taq (µL)	Water (µL)	Anneal Temp	Results
19	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	63 deg	NR
20	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	63 deg	Smear
21	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	63 deg	Smear
22	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	63 deg	Smear
23	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	63 deg	Good
24	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	63 deg	Good
25	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	65 deg	Good
26	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	65 deg	NR
27	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	65 deg	Good
28	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	65 deg	NR
29	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	65 deg	Good
30	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	65 deg	Good
31	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	67 deg	Smear
32	0.5	0.5	2.5	4 mM	0.5	0.5	17.25	67 deg	Good
33	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	67 deg	Good
34	0.5	0.5	2.5	3 mM	0.5	0.5	17.75	67 deg	Good
35	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	67 deg	Good
36	0.5	0.5	2.5	2 mM	0.5	0.5	18.25	67 deg	Good
37	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	63 deg	Smear
38	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	63 deg	Smear
39	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	63 deg	NR
40	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	63 deg	Good

Tube No.	Oligonucleotide Primers		Buffer (µL)	MgCl2	dNTPs (µL)	Taq (µL)	Water (µL)	Anneal Temp	Results
	Forward (µM)	Reverse (µM)							
41	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	63 deg	Good
42	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	63 deg	Good
43	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	65 deg	Good
44	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	65 deg	Good
45	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	65 deg	Good
46	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	65 deg	Good
47	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	65 deg	Good
48	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	65 deg	Good
49	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	67 deg	NR
50	0.1	0.1	2.5	4 mM	0.5	0.5	18.25	67 deg	Good
51	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	67 deg	Good
52	0.1	0.1	2.5	3 mM	0.5	0.5	18.75	67 deg	Smear
53	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	67 deg	Smear
54	0.1	0.1	2.5	2 mM	0.5	0.5	19.25	67 deg	Good

Appendix 2. SARDI Food Safety & Innovation *Vibrio* Culture Collection

Micro-organism	STRAIN ID	Isolated from	Location	Year
V. parahaemolyticus	Vib Is 1	Pacific Oyster	NSW	2010
V. pectenicida	Vib Is 2	Pacific Oyster	NSW	2010
V. mytili	Vib Is 3	Pacific Oyster	NSW	2010
V. vulnificus B3	Vib Is 4	Pacific Oyster	NSW	2010
Vibrio sp.	Vib Is 5	Pacific Oyster	NSW	2010
V. parahaemolyticus	Vib Is 6	Pacific Oyster	NSW	2010
V. mimicus	Vib Is 7	Pacific Oyster	NSW	2010
V. parahaemolyticus	IMVS 1425	Pacific Oyster	NSW	2010
V. parahaemolyticus	2	Pacific Oyster	NSW	2010
V. parahaemolyticus	4	Pacific Oyster	NSW	2010
V. parahaemolyticus	7	Pacific Oyster	NSW	2010
V. parahaemolyticus	10	Pacific Oyster	NSW	2010
V. parahaemolyticus	11	Pacific Oyster	NSW	2009
V. parahaemolyticus	13	Pacific Oyster	NSW	2009
V. parahaemolyticus	14	Pacific Oyster	NSW	2009
V. parahaemolyticus	17	Pacific Oyster	NSW	2009
V. parahaemolyticus	20	Pacific Oyster	NSW	2009
V. parahaemolyticus	21	Pacific Oyster	NSW	2009
V. diabolicus	23	Pacific Oyster	NSW	2009
V. parahaemolyticus	T4	Pacific Oyster	NSW	2009
V. parahaemolyticus	T6	Pacific Oyster	NSW	2010
V. vulnificus B1	R1	Pacific Oyster	NSW	2010
V. vulnificus B1	R2	Pacific Oyster	NSW	2010
V. vulnificus B1	R3	Pacific Oyster	NSW	2010
V. vulnificus B1	R4	Pacific Oyster	NSW	2010
V. vulnificus B1	R5	Pacific Oyster	NSW	2010
V. vulnificus B1	R7	Pacific Oyster	NSW	2010
V. parahaemolyticus	K	Pacific Oyster	NSW	2010
V. parahaemolyticus	P1	Pacific Oyster	NSW	2010
V. fluvialis	P7	Pacific Oyster	NSW	2010
V. parahaemolyticus	P8	Pacific Oyster	NSW	2010
V. fisheri	NB3	Pacific Oyster	NSW	2010
V. parahaemolyticus	NP8	Pacific Oyster	NSW	2010
V. agarivorans	NB9	Pacific Oyster	NSW	2010
V. fisheri	NB17	Pacific Oyster	NSW	2010
V. pectenicida	NB18	Pacific Oyster	NSW	2010
V. agarivorans	N1	Pacific Oyster	NSW	2010

Micro-organism	STRAIN ID	Isolated from	Location	Year
V. natriegens	N4	Pacific Oyster	NSW	2010
V. gigantis	N6	Pacific Oyster	NSW	2010
V. fisheri	N7	Pacific Oyster	NSW	2010
V. fisheri	N10	Pacific Oyster	NSW	2010
V. natriegens	N11	Pacific Oyster	NSW	2010
V. parahaemolyticus	N12	Pacific Oyster	NSW	2010
V. parahaemolyticus	N13	Pacific Oyster	NSW	2010
V. parahaemolyticus	N15	Pacific Oyster	NSW	2010
V. litoralis	N16	Pacific Oyster	NSW	2010
V. diazotrophicus	2190-1	Blood pipis	Retail	2011
V. mediterranei	2190-2	Blood pipis	Retail	2011
V.diazotrophicus	2190-3	Blood pipis	Retail	2011
V. mediterranei	2190-4	Blood pipis	Retail	2011
V. calviensis	2191-1	Blood pipis	Retail	2011
V. calviensis	2191-3	Blood pipis	Retail	2011
Vibrio sp.	2191-4	Blood pipis	Retail	2011
V. calviensis	2191-6	Blood pipis	Retail	2011
V. calviensis	2191-7	Blood pipis	Retail	2011
V.agarivorans	2191-9	Blood pipis	Retail	2011
V. proteolyticus	2192-1	School Prawns	Retail	2011
V. proteolyticus	2192-2	School Prawns	Retail	2011
V. coralliilyticus	2192-3	School Prawns	Retail	2011
V. proteolyticus	2192-4	School Prawns	Retail	2011
V. proteolyticus	2192-5	School Prawns	Retail	2011
V. proteolyticus	2192-6	School Prawns	Retail	2011
V. proteolyticus	2192-7	School Prawns	Retail	2011
V. proteolyticus	2192-8	School Prawns	Retail	2011
V. coralliilyticus	2192-9	School Prawns	Retail	2011
V. proteolyticus	2192-10	School Prawns	Retail	2011
V. proteolyticus	2193-1	School Prawns	Retail	2011
V. proteolyticus	2193-2	School Prawns	Retail	2011
V. proteolyticus	2193-3	School Prawns	Retail	2011
V. proteolyticus	2193-4	School Prawns	Retail	2011
V. proteolyticus	2193-5	School Prawns	Retail	2011
V. proteolyticus	2193-6	School Prawns	Retail	2011
V. proteolyticus	2193-7	School Prawns	Retail	2011
V. proteolyticus	2193-8	School Prawns	Retail	2011
V. gigantis	2194-1	Oysters	Retail	2011
Vibrio sp.	2194-2	Oysters	Retail	2011
V. mimicus	2194-3	Oysters	Retail	2011

Micro-organism	STRAIN ID	Isolated from	Location	Year
V. natriegens	2194-4	Oysters	Retail	2011
V. splendidus	2194-5	Oysters	Retail	2011
V. aestuarinus	2194-6	Oysters	Retail	2011
V. tapetis	2194-7	Oysters	Retail	2011
V. proteolyticus	2572.1	Oysters	Retail	2011
V.orientalis	2572.2	Oysters	Retail	2011
V. fortis	2572.3	Oysters	Retail	2011
V. coralliilyticus	2572.4	Oysters	Retail	2011
V. coralliilyticus	2572.5	Oysters	Retail	2011
V. coralliilyticus	2572.6	Oysters	Retail	2011
V. vulnificus B1	2572.7	Oysters	Retail	2011
V. calviensis	2572.8	Oysters	Retail	2011
V. pelagius	2572.9	Oysters	Retail	2011
V. aestuarinus	2572.10	Oysters	Retail	2011
V. rotiferanus	2573	Oysters	Retail	2011
V.mediterranei	2574.1	Oysters	Retail	2011
V. gigantis	2574.2	Oysters	Retail	2011
V. calviensis	2574.3	Oysters	Retail	2011
V. tubiashi	2574.4	Oysters	Retail	2011
V. calviensis	2574.5	Oysters	Retail	2011
V. tubiashi	2574.61	Oysters	Retail	2011
V. coralliilyticus	2574.62	Oysters	Retail	2011
V. vulnificus B1	2574.7	Oysters	Retail	2011
V. ordalii	2574.91	Oysters	Retail	2011
V. tubiashi	2574.92	Oysters	Retail	2011
V.mediterranei	2576.1	Oysters	Retail	2011
V. pelagius I	2575.2	Oysters	Retail	2011
V. parahaemolyticus	2664	Oysters	Retail	2011
V.diabolicus	2665	Oysters	Retail	2011
V. splendidus	2666	Oysters	Retail	2011
V.alginolyticus	2667	Oysters	Retail	2011
V. vulnificus	2668	Oysters	Retail	2011
V. parahaemolyticus	1808		Japan	2012
V. parahaemolyticus	1895		Japan	2012
V. parahaemolyticus	1896		Japan	2012
V. parahaemolyticus	2053		Japan	2012
V. alginolyticus	2341		Japan	2012
V. parahaemolyticus	3630		AIMS	2012

Micro-organism	STRAIN ID	Isolated from	Location	Year
V. alginolyticus	D36		AIMS	2012
V. parahaemolyticus	DY05		AIMS	2012
V. parahaemolyticus	H41		AIMS	2012
V. cincinnatiensis	K39		AIMS	2012
V. cincinnatiensis	R106		AIMS	2012
V.mediterranei			DSMZ	2012
V. aerogenes			DSMZ	2012
V. fisheri			DSMZ	2012
V. coralliilyticus			DSMZ	2012
V. furnisii			DSMZ	2012
V. harveyi			DSMZ	2012
V. parahaemolyticus			DSMZ	2012
V. vulnificus B1			DSMZ	2012
V. splendidus			DSMZ	2012
V. xuii			DSMZ	2012
V cholerae (DNA)	non-O1/non-O-139		MDU	2012
V cholerae (DNA)	non-O1/non-O-140		MDU	2012
V cholerae (DNA)	O1 ctxA positive		MDU	2012
V cholerae (DNA)	O1 ctxA positive		MDU	2012
V cholerae (DNA)	O1 ctxA positive		MDU	2012
V mimicus			MDU	2012
V vulnificus			MDU	2012
V fluvialis			MDU	2012
V fluvialis			MDU	2012
V mimicus			MDU	2012
V alginolyticus			MDU	2012
V alginolyticus			MDU	2012
V alginolyticus			MDU	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012

Micro-organism	STRAIN ID	Isolated from	Location	Year
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V parahaemolyticus			CEFAS	2012
V vulnificus			CEFAS	2012
V vulnificus			CEFAS	2012
V vulnificus			CEFAS	2012
V alginolyticus			CEFAS	2012
V alginolyticus			CEFAS	2012
V. parahaemolyticus	NR-32840		ATCC	2013
V. parahaemolyticus	NR-22029		ATCC	2013
V. parahaemolyticus	NR-32845		ATCC	2013
V. parahaemolyticus	NR-32844		ATCC	2013
V. parahaemolyticus	NR-22017		ATCC	2013
V. parahaemolyticus	NR-21017		ATCC	2013
V. parahaemolyticus	NR-31659		ATCC	2013
V. parahaemolyticus	NR-31666		ATCC	2013
V. parahaemolyticus	NR-22026		ATCC	2013
V. parahaemolyticus	NR-21994		ATCC	2013
V. parahaemolyticus	NR-22013		ATCC	2013
V. parahaemolyticus	NR-32842		ATCC	2013
V. parahaemolyticus	NR-31658		ATCC	2013
V. parahaemolyticus	NR-32841		ATCC	2013
V. parahaemolyticus	NR-31666		ATCC	2013
V. parahaemolyticus	NR-22027		ATCC	2013
V. parahaemolyticus	NR-21992		ATCC	2013
V. parahaemolyticus	NR-31665		ATCC	2013
V. parahaemolyticus	NR-31662		ATCC	2013
V. parahaemolyticus	NR-22028		ATCC	2013
V. parahaemolyticus	NR-22037		ATCC	2013
V. parahaemolyticus	NR-31665		ATCC	2013
V. parahaemolyticus	NR-21990		ATCC	2013
V. parahaemolyticus	NR-32843		ATCC	2013
V. parahaemolyticus	NR-28543		ATCC	2013
V. parahaemolyticus	NR-22002		ATCC	2013
V. parahaemolyticus	NR-31661		ATCC	2013
V. parahaemolyticus	NR-31677		ATCC	2013

Micro-organism	STRAIN ID	Isolated from	Location	Year
V. parahaemolyticus	NR-31670		ATCC	2013
V. parahaemolyticus	NR-22000		ATCC	2013
V. parahaemolyticus	NR-31671		ATCC	2013
V. parahaemolyticus	NR-31673		ATCC	2013
V. parahaemolyticus	NR-31678		ATCC	2013
V. parahaemolyticus	NR-31663		ATCC	2013
V. parahaemolyticus	NR-31676		ATCC	2013
V. parahaemolyticus	NR-62839		ATCC	2013
V. parahaemolyticus	NR-32835		ATCC	2013
V. parahaemolyticus	NR-32836		ATCC	2013
V. parahaemolyticus	NR-32837		ATCC	2013

Appendix 3. Real-Time PCR Library Results

Isolate	ID	Isolation	ctx	tlh	tdh	trh	vvh
1	*V. mediterranei*	Blood Pipi	-	-	-	-	-
2	*V. calviensis*	Blood Pipi	-	-	-	-	-
3	*V. avigorans*	Blood Pipi	-	-	-	-	-
4	*V. proteolyticus*	School Prawn	-	-	-	-	-
5	*V. coralliilyticus*	School Prawn	-	-	-	-	-
6	*V. gigantis*	Pacific Oyster	-	-	-	-	-
7	*V. mimicus*	Pacific Oyster	-	-	-	-	-
8	*V. aestuarins*	Pacific Oyster	-	-	-	-	-
9	*V. tapetis*	Pacific Oyster	-	-	-	-	-
10	*V. diazotrophicus*	Blood Pipi	-	-	-	-	-
11	*V. mediterranei*	Blood Pipi	-	-	-	-	-
12	*V. parahaemolyticus* tdh^+	Japan	-	+	+	-	-
13	*V. parahaemolyticus* trh^+	Japan	-	+	-	+	-
14	*V. parahaemolyticus* trh^+	Japan	-	+	-	+	-
15	*V. parahaemolyticus* tdh^+	Japan	-	+	+	-	-
16	*V. alginolyticus*	Japan	-	-	-	-	-
17	*V. parahaemolyticus* tdh^+ trh^+	Japan	-	+	+	+	-
18	*V. orientalis*	Pacific Oyster	-	-	-	-	-
19	*V. fortis*	Pacific Oyster	-	-	-	-	-
20	*V. coralliilyticus*	Pacific Oyster	-	-	-	-	-
21	*Vibrio sp.*	Pacific Oyster	-	-	-	-	-
22	*V. calviensis*	Pacific Oyster	-	-	-	-	-
23	*V. pelagius*	Pacific Oyster	-	-	-	-	-
24	*V. aestuarinus*	Sydney Rock Oyster	-	-	-	-	-
25	*V. rotiferans*	Sydney Rock Oyster	-	-	-	-	-
26	*Vibrio sp.*	Sydney Rock Oyster	-	-	-	-	-
27	*V. alginolyticus*	Sydney Rock Oyster	-	-	-	-	-
28	*Vibrio sp.*	Sydney Rock Oyster	-	-	-	-	-

Isolate	ID	Isolation	ctx	tlh	tdh	trh	vvh
29	*V. tubiashi*	Pacific Oyster	-	-	-	-	-
30	*V. ordalii*	Pacific Oyster	-	-	-	-	-
31	*V. alginolyticus*	Pacific Oyster	-	-	-	-	-
32	*V. parahaemolyticus*	CEFAS	-	+	-	-	-
33	*V. vulnificus*	CEFAS	-	-	-	-	+
34	*V. parahaemolyticus* tdh$^+$	CEFAS	-	+	+	-	-
35	*Vibrio sp.*	CEFAS	-	-	-	-	-
36	*V. parahaemolyticus* trh$^+$	CEFAS	-	+	-	+	-
37	*V. parahaemolyticus*	CEFAS	-	+	-	-	-
38	*V. parahaemolyticus* tdh$^+$	CEFAS	-	+	+	-	-
39	*V. parahaemolyticus* trh+	CEFAS	-	+	-	+	-
40	*V. parahaemolyticus*	CEFAS	-	+	-	-	-
41	*V. parahaemolyticus* tdh$^+$	CEFAS	-	+	+	-	-
42	*V. parahaemolyticus* trh$^+$	CEFAS	-	+	-	+	-
43	*V. parahaemolyticus*	CEFAS	-	+	-	-	-
44	*Vibrio sp.*	CEFAS	-	-	-	-	-
45	*Vibrio sp.*	CEFAS	-	-	-	-	-
46	*V. parahaemolyticus* tdh$^+$	CEFAS	-	+	+	-	-
47	*V. parahaemolyticus* tdh$^+$	CEFAS	-	+	+	-	-
48	*V. parahaemolyticus*	CEFAS	-	+	-	-	-
49	*V. parahaemolyticus*	CEFAS	-	+	-	-	-
50	*V. alginolyticus*	CEFAS	-	-	-	-	-
51	*V. alginolyticus*	CEFAS	-	-	-	-	-
52	*V. mimicus*	MDU	-	-	-	-	-
53	*V. vulnificus*	MDU	-	-	-	-	+
54	*V. fluvialis*	MDU	-	-	-	-	-
55	*V. mimicus*	MDU	-	-	-	-	-
56	*V. alginolyticus*	MDU	-	-	-	-	-
57	*V. parahaemolyticus*	MDU	-	+	-	-	-
58	*V. cincinnatiensis*	AIMS	-	-	-	-	-

Isolate	ID	Isolation	ctx	tlh	tdh	trh	vvh
59	*Vibrio sp.*	AIMS	-	-	-	-	-
60	*V. alginolyticus*	AIMS	-	-	-	-	-
61	*V. alginolyticus*	AIMS	-	-	-	-	-
62	*Vibrio sp.*	DSMZ	-	-	-	-	-
63	*V. fisheri*	DSMZ	-	-	-	-	-
64	*V. furnisii*	DSMZ	-				-
65	*V. vulnificus B1*	DSMZ	-	-	-	-	+
66	*V. xuii*	DSMZ	-	-	-	-	-
67	*V. parahaemolyticus* trh$^+$	DSMZ	-	+	-	+	-
68	*V. fisheri*	DSMZ	-	-	-	-	-
69	*V. mytili*	Pacific Oyster	-	-	-	-	-
70	*Vibrio sp.*	Pacific Oyster	-	-	-	-	-
71	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
72	*V. mimicus*	Pacific Oyster	-	-	-	-	-
73	*V. pectenicida*	Pacific Oyster	-	-	-	-	-
74	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
75	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
76	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
77	*V. parahaemolyticus* tdh$^+$	Pacific Oyster	-	+	+	-	-
78	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
79	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
80	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
81	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
82	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
83	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
84	*V. parahaemolyticus* tdh$^+$	IMVS	-	+	+	-	-
85	*Vibrio sp.*	Pacific Oyster	-	-	-	-	-
86	*V. fluvialis*	Pacific Oyster	-	-	-	-	-
87	*Vibrio sp.*	Pacific Oyster	-	-	-	-	-
88	*V. fisheri*	Pacific Oyster	-	-	-	-	-

Isolate	ID	Isolation	ctx	tlh	tdh	trh	vvh
89	*V. agarivorans*	Pacific Oyster	-	-	-	-	-
90	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
91	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
92	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
93	*V. gigantis*	Pacific Oyster	-	-	-	-	-
94	*V. litoralis*	Pacific Oyster	-	-	-	-	-
95	*V. parahaemolyticus*	Pacific Oyster	-	+	-	-	-
96	*V. diabolicus*	Pacific Oyster	-	-	-	-	-
97	*V. cholerae* non-O1/O139 (DNA)		-	-	-	-	-
98	*V. cholerae* non-O1/O139 (DNA)		-	-	-	-	-
99	*V. cholerae* O1 (DNA)		+	-	-	-	-
100	*V. cholerae* O1 (DNA)		+	-	-	-	-
101	*V. cholerae* O1 (DNA)		+	-	-	-	-
102	*C. jejuni*		-	-	-	-	-
103	*Salmonella Salford*		-	-	-	-	-
104	*Salmonella Typh.*		-	-	-	-	-
105	*E. coli*		-	-	-	-	-
106	*B. cereus*		-	-	-	-	-
107	*Y. enterocolitica*		-	-	-	-	-
108	*S. aureus*		-	-	-	-	-
109	*Salmonella Hoffitt*		-	-	-	-	-